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Isolation and Partial Characterization of a Temperature-Sensitive Mutant of Human Adenovirus Type 4

by

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A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Isolation and Partial Characterization of a Temperature-Seneitive Mutant of Human Adenovirus Type 4 submitted by Linda M. Mofford in partial fulfilment of the requirements for the degree of Master of Science in Virology.

To my parents, with love.

ABSTRACT

A random selection procedure was used to isolate a temperature-sensitive mutant of human adenovirus type 4 from nitrous acid mutagenized virus stock. The mutant displayed restricted growth at the non-permissive temperature (39°C), with a 32°C:39°C infectivity ratio of $10^4:10^1$.

Analysis of the mutant, grown at 39°C, by two-dimensional immunoelectrophoresis, showed the mutant to be defective in penton base and fiber. The mutant was, however, capable of synthesizing immunologically reactive hexon components. Temperature-shift experiments revealed detectable fiber and penton base to be present following shift-down from 39°C to 32°C. Time sequence analysis of shift-down experiments suggested a possible defect in the processing of the components, as indicated by a significant increase of immunologically detectable penton base.

Heat inactivation studies showed the mutant to exhibit an estimated 25-fold increase in thermolability at 50°C, compared to wild type HAd4 virions. The virion particles were stable in CsCl.

Analysis of viral DNA synthesis by velocity sedimentation in alkaline sucrose gradients, revealed no difference in either the extent or rate of synthesis at both the permissive and non-permissive temperatures.

The ability of the mutant to assemble virus particles at 39°C was confirmed by electron microscopy and equilibrium centrifugation. Though the virion particles produced

appeared mature, the crystalline arrays were generally of a smaller size than observed at 32°C. An overall view of the infected nuclei showed reduced viral activity, as suggested by the absence of viral inclusion bodies.

Believe nothing because a so-called wise man said it.

Believe nothing because a belief is generally held.

Believe nothing because it is written in ancient books.

Believe nothing because it is said to be of divine origin.

Believe nothing because someone else believes it.

Believe only what you yourself judge to be true.

Buddha

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ABBREVIATIONS

A	absorbance
°C	degree centigrade
bis-MSB	p-bis(o-Methylstyrl)benzene
CH_{50}	50% complement mediated hemolysis
CF	complement-fixation
cm	centimeter
cm^2	centimeter squared
CPE	cytopathic effect
CELO	chick embryo lethal orphan virus
CsCl	cesium chloride
2-D	two-dimensional immunoelectrophoresis
DEAE	diethylaminoethyl
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetra-acetate
ffu	fluorescent-focus unit
g	gravitational force
gm	grams
gm/mL	grams per millilitre
h	hour
HA	hemagglutination
HAd	human adenovirus
HE	hemagglutination-enhancement
HEC	hemagglutination-enhancement antibody consumption
HI	hemagglutination-inhibition
HIC	hemagglutination-inhibition antibody consumption

M	molarity
mA	milliampere
MEM	minimum essential media
min	minute
mL	millilitre
mm	millimeter
MOI	multiplicity of infection
NaCl	sodium chloride
NaOH	sodium hydroxide
nm	nanometer
NPT	non-permissive temperature
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PFU	plaque-forming unit
PI	post-infection
PPO	2,5-diphenyloxazole
psi	pounds per inch
PT	permissive temperature
SA7	simian adenovirus serotype 7
SC	soluble component
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
$TCID^{50}$	50% tissue culture infectious dose
Tris	tris(hydroxymethyl)aminomethane
ts	temperature-sensitive
uCi	microcurie
uL	microlitre

ug	microgram
V/cm	volts per centimeter
WT	wild type
w/v	weight per volume

I. INTRODUCTION

Adenovirus was first discovered in 1953 (Rowe, Huebner, Gillmore, Parrott, and Ward) as a passenger virus in cell cultures of adenoids used for the isolation of respiratory viruses. In the following year, Hilleman and Werner (1954) reported the presence of similar agents in respiratory secretions obtained during an epidemic of an influenza-like disease. These new agents were named adenovirus by Enders and coworkers to designate their original isolation from adenoid tissue (Enders, Bell, Dingle, Francis, Hilleman, Huebner, and Payne, 1956).

The adenovirus is a non-enveloped virion with an outer diameter of 65-80 nm. Its general structure is that of an icosahedron consisting of twenty triangular facets and twelve vertices. The outer capsid of the virion is composed of 252 capsomeres, 240 of which are hexons. The hexons are defined as those capsomeres surrounded by six neighbours (Ginsberg, Pereira, Valentine, and Wilcox, 1966). The remaining 12 capsomeres, the pentons, are located at the vertices of the icosahedron (Valentine and Pereira, 1965; Ginsberg et al., 1966). The penton is composed of both a penton base (vertex capsomere) and a fiber. The base is located within the capsid and the fiber, a rod-like projection, extends from it (Petterson and Hoglund, 1969). The penton is surrounded by five neighbouring capsomeres, referred to as the peripentonal hexons.

The inner core of the virion is rich in protein and has an inner diameter of 40-45 nm. The DNA molecule is present as a linear, duplex structure and corresponds to approximately 13% of the total virion weight (Green , Pina, Kimes, Wensink, MacHattie, and Thomas, 1976b; Van der Ed, Kestern, and van Bruggen, 1969.). Twenty percent of the viral protein is contained within this core. The coding potential of the DNA molecule is presently thought to be between 40-50 polypeptides, of an average molecular weight of 25,000.

The human adenoviruses (HAds) are presently classified according to two classification schemes, one dependent on the ability to hemagglutinate erythrocytes and one dependent on oncogenic potential. The former classification system was proposed by Rosen (1960) and divides the adenoviruses into three subgroups. Subgroup I viruses can agglutinate rhesus monkey erythrocytes, subgroup II viruses can completely agglutinate rat erythrocytes and subgroup III viruses can partially agglutinate rat erythrocytes. The latter classification system, that dependent on oncogenic potential, was proposed by Huebner (1967) and is thought to be related to the G+C content of the virus genome. This correlation of G+C content to oncogenic potential was based on the finding that whereas highly oncogenic human adenoviruses possessed a low G+C content, 48-49 per cent, weakly oncogenic HAds possessed a higher G+C content of 49-52 per cent and non-oncogenic HAds, a G+C content of

57-59 per cent (Pina and Green, 1965; Green, 1970). This relationship, however, did not apply to all the adenoviruses. Analysis of the simian adenovirus, SA7, showed it to be highly oncogenic even though it possessed a G+C content of 60 per cent (Pina and Green, 1968; Goodheart, 1971). Huebner's classification scheme divides the adenoviruses into four subgroups. Subgroup A viruses are highly oncogenic and induce tumors in most animals within weeks to months. Subgroup B viruses are weakly oncogenic and induce tumors only in a small fraction of animals within months to years. Subgroup C and D viruses are non-oncogenic (unable to induce tumors) but can transform rat embryo cells "in vitro". These two groups, C and D, are differentiated by their serologically distinct T-antigens (McAllister, Nicolson, Reed, Kern, Gilden, and Huebner, 1969). Recently, both classification systems have been questioned on the grounds that they rely on 6% or less of the viral genome (Green, Mackey, Wold, and Rigden, 1979; Wadell, 1979). Certain discrepancies between the two classification systems have also been noted. Serotypes 20, 25 and 28 have been grouped in subgroup I (Rosen's classification) but have been shown to share a common T-antigen with serotypes belonging to subgroup II (McAllister et al., 1969). Similarly, serotypes 4, 12, 18 and 31 have been classified in subgroup III (Schmidt, King, and Lennette, 1965; Norrby and Ankerst, 1969; Wadell, 1969) but whereas HAd4 shares a common T-antigen with members of subgroup I, HAd12, 18 and 31 share

a common T-antigen among themselves (Green, 1970). In view of these discrepancies, classification on the basis of DNA homology and/or polypeptide profile analysis has been proposed. The results of both methods show good correlation with Huebner's classification of oncogenicity. According to Huebner's classification system, HAd4 has been assigned to subgroup C on the basis of its non-oncogenic potential and high G+C content. However, the T-antigen of HAd4 shows immunological reactivity with that of subgroup B. The classification of HAd4 in relation to the other HAds has consequently posed some problems. In the studies by Green et al. (1979) and Wadell (1979), HAd4 was found to show no significant DNA homology with any of the other human serotypes and in addition was found to produce a unique polypeptide pattern (Marusyk and Cummings, 1978; Wadell, 1979). On the basis of these results, a separate subgroup with HAd4 as sole member has been proposed.

Studies on adenovirus infection have shown the replication cycle to consist of both an early and late phase. The early phase is defined as that preceding DNA replication. Approximately 40% of the viral genome is expressed at this time (Fujinaga and Green, 1970; Tibbetts, Pettersson, Johansson and Philipson, 1974). During the early phase, host-cell mechanisms are widely used and early gene expression is thought to involve at least three functions: the coding for viral DNA replication; the switch-over to the late phase; and possibly the switch-off of host-cell

functions (Wold, Green, and Buttner, 1978).

During the late phase of infection, host DNA synthesis is shut-down and a marked increase in the production of late viral protein occurs. Most of the protein synthesis is directed towards the production of the structural proteins, the hexons, pentons and fibers (Bello and Ginsberg, 1967; Russell and Skehel, 1972b). The production of gene products for regulatory functions is also thought to occur at this time (Wold et al., 1978) . A characteristic of the adenovirus infection is the excess production of structural proteins. Approximately 1-5% of the fibers and pentons produced and 20-30% of the hexons produced are assembled into the mature virion (White, Scharff, and Maizel, 1969; Everitt, Sundquist, and Philipson, 1971).

During replication, the viral gene products are produced in the cytoplasm of the host cell. Assembly occurs in the nucleus (Velicer and Ginsberg, 1968, 1970) and is thought to consist of a two-step process, that of capsid assembly followed by DNA insertion (Sundquist, Everitt, Philipson, and Hoglund, 1973; Ishibashi and Maizel, 1974). The functional and regulatory roles of the gene products are not known.

In the attempt to understand the mechanisms of adenovirus DNA replication and mRNA transcription, processing and translation, studies have been done on the isolation and characterization of conditional-lethal mutants. In general, most work has been done with

temperature-sensitive (ts) mutants, i.e. those that can replicate at low temperatures but not at high temperatures. The general procedure used by most workers has consisted of a mutagenization step followed by a screening procedure. The mutagenic agents which have been used are: hydroxylamine; nitrous acid; ultraviolet radiation; 5-bromodeoxyuridine; and N'-N'-nitrosoguanidine. Comparisons on the frequency of mutant isolation from author to author has been found to vary considerably. This is thought to be due to the problem of screening, which is primarily one of random selection.

The first attempt to isolate a ts mutant of adenovirus was in 1970 by Ishibashi. Ts mutants of the avian adenovirus, CELO, were isolated. In 1971, Williams and coworkers reported the isolation of ts mutants of the human adenovirus type 5 (HAd5) and on the basis of complementation experiments divided the mutants into four different complementation groups (Williams, Gharpure, Ustacelebi, and McDonald, 1971). The complementation test is designed to group together mutants expressing similar defects, i.e. mutations in the same gene(s). Since then, ts mutants of a number of human adenoviruses have been reported (Williams et al., 1971; Ensinger and Ginsberg, 1972; Suzuki, Shimojo, and Moritsugu, 1972; Weber, Begin, and Khittoo, 1975) and partial characterization of these mutants has shown a trend in the type of mutations expressed. Consequently, it has been possible to group the mutants as being either early or late.

Two complementation groups with ts defects in early genes have been identified (Ensinger and Ginsberg, 1972; Ginsberg, Ensinger, Kauffman, Mayer, and Lundholm, 1974; Williams, Young and Austin, 1974). These early mutants are unable to synthesize viral DNA and exhibit little or no synthesis of late RNA transcripts at the nonpermissive temperature (NPT). These results confirm a previous report by Bello and Ginsberg (1969) that late transcription can occur only after initiation of DNA replication. Experiments by Carter and Ginsberg (1976) further indicate that concomitant DNA replication is not required for continued late transcription. Since the two complementation groups are able to switch-off host-cell DNA synthesis, it is suspected that an early gene(s) functions in blocking host DNA replication.

Fifteen complementation groups of late mutants are presently known and all synthesize viral DNA and shut-off host DNA synthesis at NPT (Ensinger and Ginsberg, 1972; Wilkie, Ustacelebi, and Williams, 1973). These mutants can be divided into two basic groups, those that are defective in the production of any or all of the capsid proteins and those defective in some aspect of assembly.

Studies on hexon defective mutants suggest that a number of gene functions may be involved in hexon assembly, synthesis and transport. Williams et.al., (1974) reported the isolation of two groups of hexon defective mutants and characterization studies have shown that the mutants can

produce hexon monomer polypeptides but are unable to react with antibody directed against the hexon capsomere antigen (Leibowitz and Horwitz, 1975). Analysis of the monomer polypeptide showed it to be identical to wild type in size and on the basis of trypsin and chymotrypsin digest maps. Both groups were able to assemble hexon monomers at the permissive temperature (PT). A defect in the assembly of the monomer subunits into the trimer capsomeres is suspected. Mutants defective in hexon transport have also been isolated and four complementation groups are known. Partial characterization has shown the mutants to produce both normal hexon monomer polypeptides and mature trimer capsomeres (Russell, Newman, and Williams, 1972a; Russell, Skehel and Williams, 1974). The hexon, however, is not transported into the nucleus and accumulates in the cytoplasm of the host cell. All other capsid proteins are synthesized and transported normally. Complementation and recombination studies on one of these mutants, HAd5 ts147, have located the defect as being at least one cistron away from that of the hexon minus mutants (Kauffman and Ginsberg, 1976).

Mutants defective in the production of fiber and/or penton have also been isolated. In 1972, Russell et al. demonstrated the presence of three complementation groups of mutants defective in fiber antigen production (Russell et al., 1972a). This suggests that three genes are essential for the production of a functional fiber rather than two

(Dorsett and Ginsberg, 1975). Studies with fiber or penton defective mutants have also shown that neither of these components is essential for viral assembly. An examination of the morphogenesis of HAd2 mutant virions by electron microscopy showed that both penton and fiber defective mutants were able to assemble their capsids at NPT (Weber et.al., 1975; Martin, Warocquier, Cousin, D'Halluin, and Boulanger, 1978). Fiber defective mutants of HAd5 were also found to assemble capsids at NPT (Edvardsson, Ustacelebi, Williams, and Philipson, 1978). These findings support the previous experiments of Prage and coworkers (1970) which reported pentonless virions to be stable in CsCl gradients (Prage, Pettersson, Hoglund, Lonberg-Holm, and Philipson, 1970).

Preliminary characterization studies on late assembly mutants have been reported (Weber, 1976; Weber, Begin, and Carstens, 1977; Edvardsson et al., 1978), however the interpretation of the defects in relation to viral biosynthesis is not clear. Weber (1976) reported the finding of an assembly mutant defective in the processing of viral polypeptides. The mutant exhibited an accumulation of the precursor polypeptides pVI, pVII and pVIII at NPT concomitant with the absence of cleavage products. This finding correlates well with the recently proposed pathway for adenovirus assembly put forward by Philipson and coworkers (Philipson, Mathisen, Persson and Pettersson, 1978). In this pathway, Philipson proposes that adenovirus

assembly consists of four maturation stages. In the first stage, the structural proteins are assembled into the capsid skeleton, forming the empty capsid. Once the capsid is formed, insertion of the DNA and the core proteins V and VII occurs. Protein analysis of the assembly intermediates at this stage, stage II, shows that most of the structural proteins are present. No processing of the precursor polypeptides, pVI, pVII and pVIII, has, however, occurred. Stage III in the assembly pathway is marked by the accumulation of young virions in the nucleus of the host cell (Ishibashi and Maizel, 1974). These young virions exhibit all the physical and chemical characteristics of the mature virion with the exception that the precursor polypeptides are not processed. In the last stage of assembly, Stage IV, the precursor polypeptides are cleaved, resulting in the conversion of the young virions into mature virions. A diagrammatic representation of the pathway is given in Table 1.

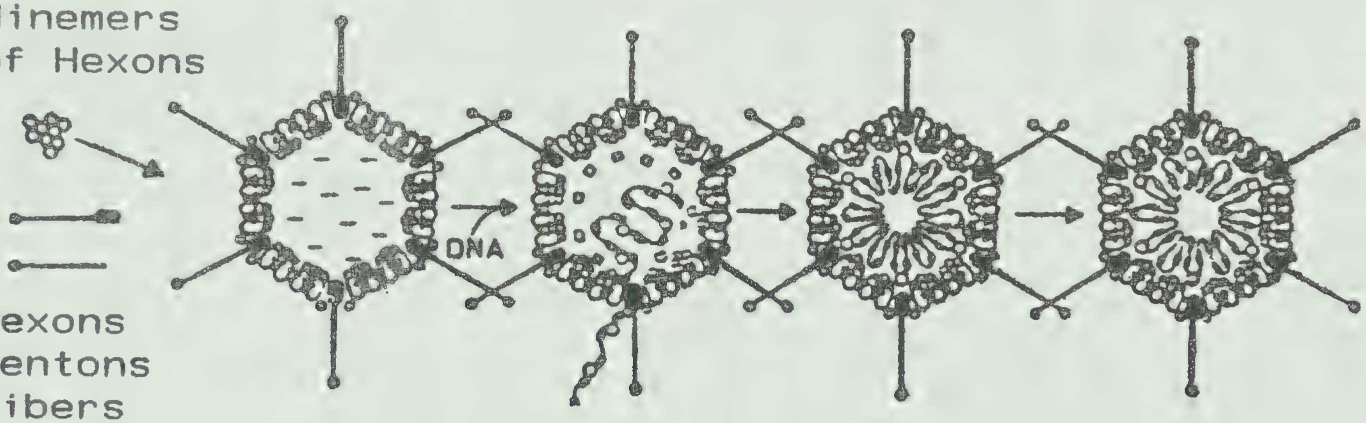
As previously stated, the assembly mutant reported by Weber (1976), HAd2 ts1, is defective in the processing of the precursor polypeptides, pVI, pVII and pVIII. Temperature-shift experiments with this mutant have shown that the processing of the precursor polypeptides is temperature-sensitive at NPT but normal at PT. In addition, if sufficient time is allotted for processing, infectious virions can be recovered at NPT. Although the nature of the defect is not known, Weber has proposed that the HAd2 ts1

Table 1[1]

Structural Proteins Synthesized in the Cytoplasm and Transported to the Nucleus

Ninemers of Hexons

Hexons Pentons Fibers and Others



Empty Capsid	Assembly Intermediate	Young Virion	Virion	
II	II	II	II	Hexon
III	III	III	III	Penton base
IIIa	IIIa	IIIa	IIIa	
IV	IV	IV	IV	Fiber
	IVa2			
	V	V	V	Core p.I.
40k?	40k?			
32-33k?	32-33k?			
pVI	pVI	pVI	VI	Hex.ass.p.
pVIII	pVIII	pVIII	VIII	Hex.ass.p.
	pVII	pVII	VII	Core p.II.
IX	IX	IX	IX	Prot.spec. for ninemers of hexons

1. Modified from Philipson et al. (1978).

gene may code for an endoprotease which is responsible for the last step of virus maturation, the conversion of young virions into mature virions through a series of maturation cleavages.

In a study by Edvardsson et al. (1978), mutants of HAd5 from four different complementation groups were found to accumulate assembly intermediates with little to no young virions produced at NPT. This finding presented the possibility of investigating two different stages in the assembly process, Stage II and III. Shift-down experiments, however, have shown that the intermediates are thermolabile and that only a fraction of the intermediates accumulated at NPT are utilized in virion assembly at PT. As a result of this finding, the relationship of the intermediates to the young virions could not be determined.

In 1977, an assembly mutant exhibiting a variety of aberrant properties in late protein synthesis was reported (Weber et al.). This mutant exhibited alterations in the production of a number of polypeptides and an inability to process others. A defect in the role of some regulatory event linking these aberrations is suspected. Its relationship in the assembly process is not known.

The use of ts mutants of adenoviruses has permitted some insight into the structure and possible functional role of certain of the viral gene products, however, much more needs to be known before the role of the viral proteins in the regulation of viral biosynthesis is understood. In this

study attempts were made to isolate and partially characterize a ts mutant of HAd4. HAd4 presents itself as an interesting candidate for investigation since it has been shown to share a number of characteristics thought to be specific for different subgroups. These findings have consequently posed interesting problems in both its classification and relationship to the other HAds. Originally HAd4 was classified in subgroup III (Rosen's classification) on the basis of its ability to partially agglutinate rat erythrocytes. Since it was found to be nononcogenic and possessed a high G+C content, it was also classified in subgroup C according to Huebner's classification system. The immunological reactivity of the HAd4 T-antigen and capsid subunits, however, showed it to express a close relationship with the members of subgroup B. This relationship with subgroup B was also demonstrated with HAd4 pathogenicity, the type of CPE expressed by HAd4 and the kinetics of HAd4 multiplication and neutralization. In addition, HAd4 has been shown to induce dodecon synthesis, a characteristic of subgroups B and D. Despite these similarities of HAd4 with the different subgroups, B, C, and D, DNA homology studies have shown that the DNA of HAd4 is distinct from the DNAs of any of the other HAds (Green, 1979). In addition, polypeptide characterization of HAd4 has shown it to produce a unique polypeptide profile (Marusyk and Cummings, 1978; Wadell, 1979). In view of these differences, the isolation and characterization of ts

mutants of HAd4 should offer interesting comparisons with that of other HAd ts mutants.

II. METHODS AND MATERIALS

A. CELL CULTURES

HEp-2 (human oral carcinoma) and HeLa (human cervical carcinoma) cells were obtained commercially (Flow Laboratories, Inc., Inglewood, Ca.). The cells were grown in glass culture vessels (Blake bottles) and when 90-100% cell confluency was reached, the medium was removed and the cells washed with 2 mL of 0.25% (w/v) trypsin (Difco). A further 2 mL of trypsin (same concentration) was added and the cells incubated at 37°C until they detached from the glass surface. The cells were then diluted in medium and dispensed into Blake bottles where they were either maintained until re-passage or infected with virus when the monolayer reached 75% confluency.

B. CELL CULTURE MEDIUM

Minimum essential medium (MEM, Eagle's Modified Auto Pow, Flow Laboratories, Inc., Inglewood, Ca.) was dissolved in distilled water and autoclaved for 15 min at 15 psi, 120°C. Prior to use, the medium was supplemented with a final concentration of 3% sterile calf serum, 0.002 M glutamine and 0.1% (w/v) sodium bicarbonate. Penicillin-G (100 units/mL, final concentration) and streptomycin sulfate (200 ug/mL, final concentration) were also added. With the exception of the calf serum, all supplements were filter sterilized prior to use.

C. VIRUS

Human adenovirus serotype 4 was the kind gift of Dr. G. Wadell, Dept. of Virology, Karolinska Institute, Stockholm, Sweden.

D. VIRUS PROPAGATION

Ten to twenty Blake bottles of HEp-2 cells were routinely used for virus propagation. Prior to infection, the tissue culture medium was discarded and the cells infected with 10 mL of purified virus suspension (5 ffu/cell, see section F) for 30-60 min at 37°C. Following adsorption, 100 mL of tissue culture medium was added and the cells incubated at 37°C until complete cytopathic effect (4+ CPE, 72-96 h) was observed. The medium containing the infected cells was pooled and centrifuged at 800 g for 15 min. The resulting pellet was re-suspended in PBS and stored at -20°C until further use.

E. VIRUS PURIFICATION

Virus was purified by a three-step process. The process consisted of a fluorocarbon extraction procedure followed by centrifugation in CsCl gradients. The virus-infected cell pellet was suspended in a mixture of 1 part Freon 113 (trichlorotrifluoroethane; Dupont, Maitland, Ontario) to 2 parts PBS and homogenized in a Sorvall Omni-Mixer (Dupont Instruments, Wilmington, Delaware) for a

period of 4 min, with a 1 min cooling period between each min of mixing. The resulting suspension was centrifuged at 800 g for 15 min and the supernatant pooled and layered on the top of a step-gradient of CsCl consisting of 5 mL of each of the following buoyant densities: 1.40 gm/mL, 1.32 gm/mL and 1.20 gm/mL. Centrifugation was carried out at 55,000 g for 90 min in a SW27 rotor. Following centrifugation, the region above the opalescent virus band was collected as "soluble components" (SC) and stored at -20°C. The virus band was then removed and further purified by equilibrium centrifugation in CsCl, buoyant density 1.339 gm/mL. Centrifugation was carried out at 150,000 g for 24-48 h using a SW50 rotor. The resulting virus band was then collected, dialyzed overnight against Tris-saline buffer (0.02 M Tris-HCl, 0.05 M NaCl, pH 7.4) and either stored at 4°C or mixed with glycerol (50% final concentration) and stored at -20°C until further use. All centrifugations were done using a Beckman L5-75 preparative ultracentrifuge.

F. VIRUS ASSAY

Viral infectivity was determined by three methods: fluorescent focus assay, $TCID^{50}$ (50% tissue culture infectious dose) assay and plaque-forming assay.

a) Fluorescent Focus Assay

The fluorescent focus assay used was a modification of the technique described by Thiel and Smith (1967). HeLa cells were seeded at a concentration of 1.5×10^6 cells/mL

into disposable tissue culture plates (60 mm diameter, Corning Glass Works, Corning, N.Y.) and maintained in medium at 37°C in a 5% carbon dioxide atmosphere until 90-100% confluent (24-36 h). The cells were then washed twice with 5-8 mL of PBS and inoculated with 0.1 mL of a 10-fold serial dilution series of the virus sample. All dilutions were done in duplicate and two uninoculated cultures were used as negative controls. The virus was allowed to adsorb for 1-2 h (1 h at 37 and 39°C, 2 h at 32°C) during which time the plates were gently rotated every 15-30 min. Following adsorption, 10 mL of maintenance medium was added to each of the plates and the cells incubated for either 48 h at 37 and 39°C or 72 h at 32°C. The medium was then discarded and the cells fixed with 5 mL of cold methanol for 30-60 min at -20°C. The plates were air dried and either stained immediately by the immunofluoresence staining procedure (see section G) or stored at -20°C . The number of fluorescent focus units per mL was calculated as described by Thiel and Smith (1967). A single fluorescent focus unit is defined as representing one infected cell.

b) $TCID^{50}$

Tissue culture tubes were inoculated with 2 mL of HeLa cells in MEM at a concentration of 5 x 10^5 cells/mL. The cells were incubated at 37°C until 75-80% confluent (24 h). The medium was then discarded and the cells infected with 0.1 mL of a 10-fold dilution series of the virus sample. All dilutions in the series were done using a sample size of 2

tissue culture tubes per dilution. The virus was allowed to adsorb for 1-2 h (1 h at 37 and 39°C, 2 h at 32°C) with occasional rotation, followed by the addition of 3 mL of medium. The cells were incubated at the appropriate temperature with the medium routinely changed every 3-5 days for the length of the assay. The assay period varied depending on the incubation temperature used. A 10 day assay period was used for cells incubated at 37 and 39°C versus a 14 day assay period for cells incubated at 32°C. Virus titres were calculated on the final day of incubation by the Reed and Muench method for determination of the 50% endpoint (Reed and Muench, 1938).

c) Plaque-Forming Units (PFU)

Disposable tissue culture plates (60 mm diameter, Corning Glass Works, Corning, N.Y.) were seeded with 3×10^6 HeLa cells and maintained in medium at 37°C in a 5% carbon dioxide atmosphere until 95-100% confluent. The cell monolayers were then washed twice with 5 mL PBS and inoculated with 0.1 mL of the virus sample appropriately diluted in PBS. The plates were rotated gently at 30 min intervals throughout an adsorption period of 120 min at 32°C. Following the adsorption period, the cell monolayers were overlaid with an initial volume of 5 mL of agar medium prepared as follows:

A = 1.3% Noble Agar (Difco) in distilled water,, kept at 43°C.

B = 2X MEM, without phenol red, containing 2X

concentration of medium additives plus 0.4 mM arginine and 3% calf serum, kept at 37°C. Occasionally, the agar medium was also supplemented with 25 mM magnesium chloride (Williams, 1970; Raskas, Prage, and Schlesinger, 1972; Rouse and Schlesinger, 1972).

The overlay consisted of 1 part A plus 1 part B medium. Once the agar solidified, the plates were inverted and incubated at 32°C in a 5% carbon dioxide atmosphere. Three days postinfection, 5 mL of the same overlay was added and the plates incubated until plaques could be observed under the light microscope (10-14 days). The plaques were then either picked with a pasteur pipette and stored in 0.5 mL medium at -35°C, or an additional 5 mL of the same overlay containing 1:10,000 neutral red was added to the monolayer and the plates examined for the development of plaques 24-48 h later.

G. IMMUNOFLUORESCENCE STAINING PROCEDURE

Infected cells fixed with cold methanol (-20°C) and air dried were washed with 5 mL PBS and incubated with 0.1 mL of HAd4 antiserum (1:200 dilution in PBS) for 60 min at 37°C. The cells were washed three times with PBS to remove excess antisera and re-incubated for another 60 min at 37°C with 0.1 mL fluorescein-conjugated sheep anti-rabbit immunoglobulin (1/40 dilution in PBS; Cappel Laboratories, Cochranville, P.A., U.S.A.). The cells were washed twice with PBS followed by a final wash with distilled water to

remove excess salt and then microscopically examined for the presence of fluorescent-foci. All microscopic examinations were done using a Zeiss Epi-fluorescence microscope.

H. PREPARATION OF HYPERIMMUNE SERUM

Purified wild type HAd4 was prepared by two CsCl equilibrium centrifugations. The virus sample was dialyzed overnight against distilled water (4°C) after which 0.5 mL of the virus was emulsified with an equal volume of complete Freund adjuvant. The mixture was injected intramuscularly into rabbits. One month following the first injection, a one mL booster of purified HAd4 (10^{10} ffu/mL) was injected intravenously. The rabbits were exsanguinated ten days later and the sera collected and stored at -20°C.

I. SEROLOGICAL ASSAYS

The following serological assays were performed as described by Norrby and Skarret (1967a).

a) HEMAGGLUTINATION (HA) ASSAY

Serial two-fold dilutions of virus or soluble components were prepared in PBS using a microtitration system (Cooke Engineering Company, Alexandria, Va., U.S.A.). To each 25 uL of diluted antigen, 25 uL of 1% rat erythrocytes, prepared in PBS, was added. The mixture was incubated at 37°C for 60 min and the titre read as the highest dilution producing complete agglutination of erythrocytes.

b) HEMAGGLUTINATION-INHIBITION (HI) ASSAY

Serial two-fold dilutions of HAd4 antiserum, against whole, disrupted virions were prepared in PBS using the microtitration system described above. To each dilution of antiserum, 4 HA units of antigen in 25 uL was added. The mixture was incubated for 1 h at 37°C. Following incubation, 50 uL of 1% rat erythrocytes prepared in PBS were added per well and the mixture incubated as before. The HI titre was read as the highest dilution of antiserum causing complete inhibition of agglutination.

c) HEMAGGLUTINATION-INHIBITION ANTIBODY CONSUMPTION (HIC) ASSAY

Serial two-fold dilutions of the samples to be analyzed were prepared in PBS as described in the HI assay. To each dilution, 25 uL of 2 HI units of HAd4 antiserum was added. After incubation for 30-60 min at 37°C, 25 uL of 2 HA units in the form of stock SC material was added per well. The mixture was incubated as above, followed by the addition per well of 50 uL of a 1% rat erythrocyte suspension prepared in PBS. The cells were then allowed to settle for 30-60 min at 37°C. The highest dilution of the sample adsorbing the added HI antibodies, i.e. exhibiting maximal or clear-cut partial agglutination, was read as containing one HIC unit.

d) HEMAGGLUTINATION-ENHANCEMENT (HE) ASSAY

Serial two-fold dilutions of virus or soluble components were prepared in PBS using the microtitration system mentioned above. To each 25 uL of diluted antigen, 25 uL of HAd6 antiserum was added and the mixture incubated at

37°C for 30-60 min. Fifty uL of 1% rat erythrocytes (in PBS) was then added to each well and the mixture incubated as before. The HE titre was read as the highest dilution producing complete agglutination of erythrocytes.

e) HEMAGGLUTINATION-ENHANCEMENT ANTIBODY CONSUMPTION (HEC) ASSAY

The technical procedure followed for the HEC assay was similar to that for the HIC assay. To serial two-fold dilutions of test samples, 25 uL of 2 HE units of anti-adenovirus type 6 serum was added. The mixture was incubated for 30-60 min at 37°C, followed by the addition, per well, of 25 uL of 2 units of incomplete HAd5 hemagglutinin. After a 30-60 min incubation period at 37°C, 50 uL of 1% rat erythrocytes prepared in PBS was added to each well. The cells were then incubated as before for 30-60 min. The HEC titre was read as the highest dilution exhibiting a pattern of non-agglutinated red blood cells.

Performance of this test required that all test samples did not contain complete HA. Consequently, prior to performing the HEC assay, all samples were screened for the absence of HA activity.

J. COMPLEMENT FIXATION (CF) TEST

Complement fixation tests were performed according to the microtitration technique described by Bradstreet and Taylor (1962). Complement and haemolysin were obtained commercially from Behring Diagnostics (Montreal, Quebec) and

M.A. Bioproducts (Walkerville, Maryland), respectively, and their appropriate titres determined by means of a checkerboard titration in which both complement and haemolysin concentrations were varied. HAd6 antiserum prepared in rabbits against whole disrupted virions was used as the stock antiserum at a standard concentration of 2 units. Prior to use, the serum was inactivated for 30 min at 56°C and tested for anticomplementarity. Serial two-fold dilutions of the test samples were then prepared. To each dilution, 2 units of HAd6 antiserum was added followed by the addition of 4 CH_{50} units of complement. The mixture was covered and incubated at 4°C. Following incubation, the samples were kept at 37°C for 30 min. A 2% suspension of sheep red blood cells (Armand-Frapier, Laval, Quebec) sensitized with haemolysin (1/100 dilution) was then added to each well. The mixture was incubated at 37°C for 50 min with intermittent shaking every 15-20 min. The cells were then allowed to settle at 4 °C for 30 min. Complement fixation titres were read as the highest dilution of test sample causing 50% lysis of the red blood cells. Appropriate controls were incubated with every test.

K. GROWTH CHARACTERISTICS

HeLa cell monolayers were infected with wild type HAd4 at a multiplicity of infection (MOI) of 5 ffu/cell. After adsorption at 37°C for 1 h, the monolayers were washed with PBS and maintenance media added. Duplicate tubes were

incubated at 32, 37, and 39°C. The cell cultures were harvested at various intervals postinfection at which time the cells were pelleted and resuspended in 1 mL of maintenance media. Following three cycles of freeze-thawing, virus yields were determined by assaying the resulting lysate for the presence of fluorescent-foci forming activity. The assay procedure followed was as described in Methods and Materials.

L. NEUTRALIZATION ASSAY

A modification of the neutralization assay as described by Rovozzo and Burke (1973) was used. Purified HAd4 was appropriately diluted in PBS to yield 30-50 ffu/field as determined in confluent cell monolayers of HeLa cells prepared in disposable tissue culture plates. The diluted virus sample was then mixed with an equal volume of serial two-fold dilutions of HAd4 antiserum directed against whole virions and incubated at room temperature for 2 h. The virus-antiserum mixtures were then titred at 32°C according to the fluorescent focus assay outlined previously. The neutralization titre was read as the highest antiserum dilution completely inhibiting the production of ffu.

M. SCREENING AND MUTAGENIZATION PROCEDURES

The screening and mutagenization procedures followed in the course of the study were as described by Williams et al. (1971). Wild type HAd4 which had been plaque purified twice

was mutagenized by treatment with either nitrous acid or hydroxylamine. In both cases the screening procedure consisted of randomly picking plaques and testing for growth at both the permissive and non-permissive temperatures, 32 and 39°C, respectively. Picked plaques were dispensed in 0.5 mL maintenance media and freeze-thawed three times after which the plaque-associated virus was either tested directly for growth at both temperatures or first allowed to grow at 32°C in order to increase titre, followed by testing for growth at 39°C. All testing was done using 25 cm^2 Falcon flasks (Corning Glass Works, Corning, N.Y.) seeded with HeLa cells at 80-90% confluency.

a) Nitrous Acid Mutagenization

To prepare a stock of mutagenized virus by nitrous acid treatment, equal volumes of 1.4 M sodium nitrite in 2 M acetate buffer, pH 4.6, and WT HAd4 (10^5-10^8 ffu/mL) were incubated for 5-7 mins at room temperature. The reaction was stopped by the addition of 4 volumes of cold 1 M Tris-HCl, pH 7.9. The stock was appropriately diluted and immediately assayed for the production of plaques at 32°C. All plaques were located with an inverted microscope, marked, picked with a pasteur pipette and screened according to the protocol outlined above. This mutagenization procedure resulted in a 2 log drop in titre.

b) Hydroxylamine Mutagenization

Preparation of a stock of mutagenized virus by hydroxylamine treatment consisted of incubating equal

volumes of WT virus (10^5-10^8 ffu/mL) and 2 M hydroxylamine-HCl in PBS. The reaction mixture was incubated at 37°C and samples removed at 1 h intervals for a 5 h period. The samples were diluted 1:5 in PBS and dialyzed overnight against the same buffer to remove any residual hydroxylamine. Serial 10-fold dilutions of the samples were prepared, assayed for plaque development and later screened as outlined above. This procedure resulted in a 2 log drop in titre.

N. HEAT INACTIVATION

Heat inactivation of the virus samples was determined according to the method outlined by Weber et al. (1975). Crude virus samples grown at 32°C were dispensed in 0.2 mL volumes into replicate tubes and immersed in a 50°C water bath. At time intervals of 0, 8, and 16 min, duplicate samples were removed and cooled immediately on ice. The infectivities of both heated and unheated virus samples were determined by the fluorescent-focus assay.

O. IMMUNOELECTRPHORESIS

A modification of the procedure described by Weeke (1973) was used. Strips of GelBond film (FMC Corporation, Rockland, Maine; (10 cm^2)) were covered with 15 mL of melted 1% (w/v) agarose (Bio-Rad Laboratories) in electrode buffer (0.05 M Tris-HCl and 0.4 M glycine), pH 8.0. After solidification, 3 or 4 mm sample wells were punched into the

gel using a template guide and the appropriate punch. Ten to 40 uL of sample were added to each well and the plate placed in a horizontal electrophoresis apparatus (Bio-Rad Laboratories, Model 1400 Electrophoresis Cell) with "Telfa" wicks for the buffer-gel connection. First dimension electrophoresis was carried out at 15 V/cm for 90 min with water cooling.

Following first dimension electrophoresis, the agarose gel was divided into strips, each strip corresponding to one well and containing the area of the migrated antigen. The strip was transferred to a second plate and aligned along one edge. The remaining part of the plate was filled with 10 mL of 1% agarose containing 25 uL of antiserum. The plate was then electrophoresed in the second dimension (at right angles to the first dimension) at 25 V/cm for 3.5 h. At the end of the run the gel was pressed for 30 min under a layer of filter paper and a 3 cm layer of blotting paper. The gel was then dried with a hot air blower and stained for 15 mins with 0.2% Coomassie Brillant Blue R dissolved in a solution of 50% methanol, 10% acetic acid and 40% distilled water. The gel was destained using the same solution without stain.

P. PREPARATION OF SOLUBLE COMPONENTS FROM HAd4 AND TS MUTANT STOCKS FOR 2-D ANALYSIS

Disposable tissue culture plates seeded with HeLa cells (90-100% confluent) were infected with WT Ad4 or the HAd4

ts1 mutant. After appropriate incubation at 32 and/or 39°C, the infected cells were scraped off the plate and centrifuged at 800 g for 10 min. The resulting cell pellet was washed once in PBS, re-centrifuged (800 g, 10 min) and dispensed into a final volume of 1 mL PBS. The cells were then freeze-thawed three times and freon extracted as described previously. The resulting supernatant was either stored at -20°C or immediately assayed by two-dimensional immunoelectrophoresis and for HA titre.

Q. LARGE-SCALE PREPARATION OF HAd4 SOLUBLE COMPONENT (SC) MATERIAL

Large-scale preparation of SC was done according to the method described by Boulanger and Puvion (1973). Soluble components from 100-150 Blake bottles of HEp-2 cells infected with WT HAd4 were prepared as described previously. The SC material was pooled and mixed with a saturated solution of ammonium sulphate at 4°C, to a final concentration of 55% saturation. The mixture was left at 4°C overnight and the resulting precipitate centrifuged at 500 g for 20 min (4°C). The pellet was resuspended in 0.05 M sodium phosphate buffer, pH 6.8, and dialyzed for 48-72 h at (4°C) against the same buffer. The dialyzing buffer was changed 3-5 times throughout this period. Following dialysis, the viral antigen was centrifuged at 110,000 g for 2 h in order to clear the solution of any possible incomplete, low-density virus particles. The final

supernatant was then concentrated to the appropriate volume with polyethylene glycol 20,000, dialyzed for 24 h against 0.05 M sodium phosphate buffer, pH 6.8, and stored at -20 °C for further separation.

R. SEPARATION OF SOLUBLE COMPONENTS BY ION-EXCHANGE CHROMATOGRAPHY

DEAE-Sephadex A-25 (Pharmacia, Uppsala, Sweden) was prepared as a 10% (w/v) suspension. The slurry was washed with several volumes of 0.05 M Tris-HCl, pH 6.8, and packed by gravity in a 1 x 30 cm column. A three mL layer of a 5% suspension of Sephadex G-25(Pharmacia, Uppsala, Sweden), prepared as above, was layered on the top of the column and the column washed with 25 mL of equilibrating buffer (0.05 M Tris-HCl, pH 6.8). Samples of 2-4 mL of stock HAd4 SC material, dialyzed against the eluting buffer, were then layered on the top of the column and eluted with 125 mL of a linear gradient of either 0-0.5, 0-0.4, or 0-0.3 M NaCl in 0.05 M Tris-HCl, pH 6.8. Conductivity of each fraction was determined using a conductivity salt bridge apparatus (Model 31, Yellow Springs Instruments, Yellow Springs, Ohio). Molarity was determined by use of a computer-generated 'best-fit' (least squares) straight line programme.

S. SEPARATION OF SOLUBLE COMPONENTS BY RATE ZONAL CENTRIFUGATION

Separation of the soluble components was achieved by a series of zonal centrifugations using a Beckman L5-75 preparative ultracentrifuge and a SW40 rotor. Stock HAd4 soluble component material, prepared as previously described, was layered on the top of a linear 5-20% sucrose gradient and centrifuged at 77,000 g for 16 h at 4°C. Fractions were collected dropwise from the bottom of the tube and assayed for absorbance readings at 280 nm, using a Gilford Spectrophotometer 250. Each fraction was screened for HA activity. Those fractions exhibiting positive hemagglutination were pooled, concentrated with polyethylene glycol 20,000, and dialyzed overnight against 0.05 M sodium phosphate buffer, pH 6.8. This procedure was routinely followed for all pooling, concentrating, and dialyzing of fractions. Final purification of the HA activity was achieved by subjecting the sample to a second centrifugation, performed under identical conditions as outlined above. The remaining fractions, those exhibiting negative HA activity, were concentrated and dialyzed according to routine procedure and recentrifuged on a linear 5-20% sucrose gradient at 77,000 g for 35 h. The gradient was collected as before with individual populations of the soluble components being identified serologically by HE, CF, HEC and/or HIC assays. Final purification was achieved by recentrifugation of the individual populations at 77,000 g

for 35 h.

T. DNA ANALYSIS

A modification of the method described by Weber et al. (1975) was used in this study. Confluent monolayers of HeLa cells grown in 75 cm^2 tissue culture flasks (Corning Glass Works, Corning, New York) were inoculated with WT HAd4 and the HAd4 ts1 mutant at a multiplicity of infection of 10 ffu/cell. The cells were incubated at 32 and 39°C and labelled 20 and 40 h later, respectively, with 5 uCi/mL of (^{3}H)-thymidine. At 40 h postinfection, the cells were scraped off with a rubber policeman and harvested by centrifugation at 800 g for 15 min. The cells were then resuspended in cold Tris-saline buffer, pH 7.4 (0.02 M Tris-HCl, 0.05 M NaCl). Cultures incubated at 32°C were harvested in a similar manner at 65 h postinfection. The cells were then layered on the top of a linear 5-20% alkaline sucrose gradient in 0.3 M NaOH, 0.7 M NaCl and 0.001 M Na EDTA. A lysing layer (0.2 mL) consisting of 0.5 M NaOH and 0.05 M Na EDTA was carefully applied on top of the samples. After standing for 16 h at 4°C, the gradients were centrifuged in a Beckman L7-75 preparative ultracentrifuge for 5 h at 150,000 g using a SW40 rotor. Fractions were collected dropwise from the top of the gradient after which 5 uL of each fraction was collected on a filter (Whatman No. 1) and assayed for radioactivity. The filters were washed with a 10% cold TCA solution followed by methanol. The

filters were air dried and counted in a toluene based scintillation fluid, containing Triton X-100, 2,5-diphenyloxazole (PPO) and p-bis(o-Methylstyryl)benzene (bis-MSB). Counts were determined using a LKB 1215 RacKbeta Liquid Scintillation Counter (Wallac Oy, Turku, Finland).

U. VIRION STABILITY IN CESIUM CHLORIDE

HeLa cell monolayers were infected with virus at a multiplicity of infection of 5 ffu/cell. The cells were incubated at either 39 or 32°C and labelled at 20 and 40 h postinfection, respectively, with 5 uCi/mL of (^{3}H)-thymidine. The label was left on the cells until the cells were harvested in PBS, 2 and 3 days after labelling at 39 and 32°C. The cells were then lysed and freon-extracted as described previously. The resulting material was layered on the top of a cesium chloride solution (1.339 gm/mL) and centrifuged at 150,000 g for 24 h using a SW50 rotor in a Beckman L7-75 preparative ultracentrifuge. Following centrifugation, fractions were collected dropwise from the bottom of the gradient. The density of the cesium chloride was determined by refractometry, using an Abbe Refractometer (Erma Optical Works Ltd., Tokyo, Japan). Radioactivity was determined as 10% cold TCA-precipitable counts in toluene based scintillation fluid. Details of the procedure followed for assaying radioactivity are as described in the section for DNA synthesis.

V. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

SDS-polyacrylamide slab gels were prepared using a modification of the method described by Maizel (1971). The polyacrylamide slab gels were prepared in a Bio-Rad Model 220 electrophoresis apparatus (150 x 100 x 1.5 mm) with a 3% stacking gel layered on the top of a 13% resolving gel (acrylamide-bisacrylamide ratio of 30:0.8). Twenty mL of the resolving gel was added to the apparatus and allowed to polymerize for 30-60 min. A slotted comb was then inserted into the apparatus and 10 mL of the stacking gel added. Following polymerization of the gel, the comb was removed and the formed slots filled with electrode buffer.

Samples for analysis were prepared as outlined below. The protein concentration of each sample was determined by the Bio-Rad protein assay system described by Bradford (1976). Fifty to 100 ug of protein of each sample was drop-dialyzed (Marusyk and Sergeant, 1980) against 1% ammonium acetate at room temperature. After dialysis, the samples were collected and dissociation buffer added to a final concentration of 0.05 M Tris-HCl, pH 7.6, 1% sodium dodecyl sulphate, 0.1% beta-mercaptoethanol, 10% glycerol and 0.002% bromophenol blue. The samples were heated at 98°C for 2 mins. and then layered onto the gel. Electrophoresis was carried out at a constant current of 30 mA for 220 min. At the end of the electrophoresis, the slab gel was removed and stained overnight in 0.2% Coomassie Brillant Blue R solution and destained as described above.

The molecular weight of the polypeptides of each sample was determined by comparison with the relative mobility of samples of known molecular weight, i.e. HAd2 polypeptides and/or standard polypeptide markers electrophoresed in the same gel. In this sudy the commercially available standard polypeptide markers used were: phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin, and alpha-lactalbumin (Pharmacia Fine Chemicals, Piscataway, N.J.). The molecular weights of these standard polypeptides are, 94,000, 67,000, 43,000, 30,000, 20,100, and 14,400, respectively. Molecular weight calculations were done using a computer generated "best-fit" (least squares) straight line programme.

W. VIRION ASSEMBLY

HeLa cell monolayers were infected with wild type HAd4 and the HAd4 ts1 mutant at a multiplicity of infection of 5 ffu/cell. The infected cells were incubated at 37, 39, or 32°C for 16, 43, and 90 h, respectively. Following incubation, the maintenance medium was removed and the cells fixed with 3% cold glutaraldehyde in 0.1 M phosphate buffer, pH 7.2. After fixing for 15 min, the cells were gently scraped off the petri dish with a rubber policeman, centrifuged in glass conical tubes for 20 min at 800 g and postfixed in 1% osmium tetroxide for 2 h. The samples were then dehydrated in an alcohol gradient of 50-100% ethanol and embedded in Epon 812. Polymerization was done at 60°C

for 2 days. Thin sections were cut with a Porter-Blum microtome and stained with uranyl acetate followed by lead citrate. Sections were examined on a Siemens ELMISKOP 102, electron microscope.

X. VIROLOGICAL TECHNIQUES

All standard virological techniques used in the course of the study were performed according to the methods outlined by Rovozzo and Burke (1973).

Y. BUFFER SOLUTIONS

Buffer solutions required for the course of this study were prepared from tables listed by Gomori (1955).

Z. MANUSCRIPT PRODUCTION

This thesis was produced by the Textform programme of the Michigan Terminal System, using a Xerox 9700 page printer.

III. RESULTS

A. VIRUS ASSAY

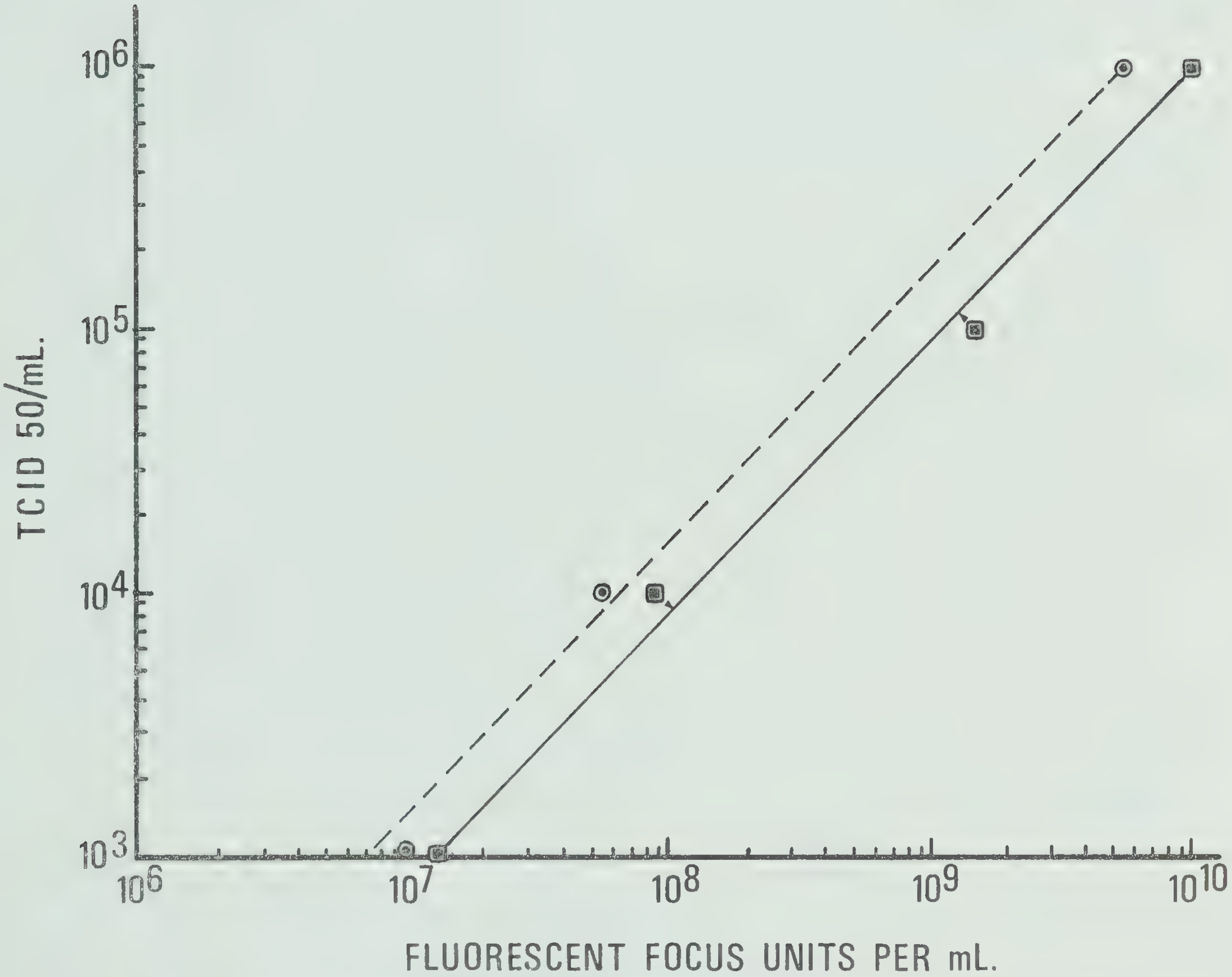
Attempts were made to assay HAd4 by the plaque assay method using both HeLa and HEp-2 cell lines. This assay, however, proved to be unsatisfactory, since the plaques produced by the HAd4 virus stock were found to be of a pin-point size which made visual location of the plaques difficult. Attempts to enhance plaque formation by either subjecting the cells to longer incubation periods or by the addition of magnesium chloride and/or arginine to the assay media were examined, but were found to be ineffective. As a result, all viral titers were determined by either the fluorescent-focus (ff) assay or by the $TCID_{50}$ assay. Comparison of these two assays showed a linear relationship to exist between them, as shown in Figure 1.

Since the viral titers were to be determined at three different temperatures, 32, 37, and 39°C, a standardization of the ff and $TCID_{50}$ assays was necessary. The minimum incubation time required to yield similar titers at each of the three temperatures was first examined by comparing the production of ffu at different incubation times. The results, as given in Table 2, showed that a 72 h incubation period at 32°C yielded a similar titer to that of a 48 h incubation period at 37 or 39°C. In addition, no significant increase in titer was observed if the incubation periods were extended an additional 24 h.

Figure 1.

Relationship Between the Fluorescent Focus and $TCID_{50}$ Assays

The respective assays were carried out as described in Methods and Materials. The results of two separate studies are shown.



TCID 50/mL.
10^6
10^5
10^4
10^3
10^6
10^7
10^8
10^9
10^10
FLUORESCENT FOCUS UNITS PER mL.

Table 2

Standardization of the FF Assay at 32, 37, and 39°C

Fluorescent-focus units were assayed as described in Methods and Materials. The titers were recorded as the average count of 10 fields per tissue culture plate with 2 tissue culture plates being used per dilution. The adsorption periods varied depending on the temperature of incubation; a 1 h adsorption period was used for 37 and 39°C and a 2 h adsorption period for 32°C.

N.D. - not done

Incubation Temperature	Incubation Period (Hours)		
	48	72	120
32°C	ND	2.17×10^7	1.76×10^7
37°C	1.14×10^7	1.16×10^7	ND
39°C	1.38×10^7	1.28×10^7	ND

With the $TCID_{50}$ assay, the length of incubation required to yield similar titers was also found to be dependent on the temperature of incubation. In general, using a 10-day incubation period at 37°C as the standard, equivalent titers at 39 and 32°C were obtained after 10 and 14 days, respectively.

B. PURIFICATION OF HAd4 SOLUBLE COMPONENTS

I. Separation of Components

HAd4 soluble components (SC) were prepared as described in Methods and Materials. Various techniques have been described for the separation of adenovirus soluble components (Norrby and Wadell, 1967b; Wadell and Skaaret, 1967; Wilcox and Ginsberg, 1961). In this study, both ion-exchange chromatography and rate zonal centrifugation techniques were used.

a) Ion-Exchange Chromatography

Separation of the HAd4 soluble components by ion-exchange chromatography was not successful. Initial trials using a linear NaCl salt gradient of 0-0.5 M, resulted in only limited separation of the soluble components. Shallower gradients of 0-0.3 M and 0-0.4 M, were examined in an attempt to enhance separation, but again limited separation resulted. In each case, the soluble components eluted in close proximity to each other, often

eluting at overlapping salt concentrations. The situation was further complicated by the presence of excess hexons in the stock material, resulting in contamination of the other soluble component populations.

The inability to sufficiently separate the soluble components made the ion-exchange chromatography technique unsuitable for the purpose of isolating relatively pure soluble component populations. Furthermore, the dilution effect introduced by the technique posed serious problems both in the recovery and identification of the minor component populations and at times resulted in a complete loss of these components.

b) Rate Zonal Centrifugation

As a result of the difficulties with ion-exchange chromatography, separation of viral soluble components was attempted by rate zonal centrifugation. This technique gave reproducible results with minimal loss of material. A schematic representation of the method used is given in Figure 2.

The HAd4 SC stock material, prepared as described in Methods and Materials, was layered onto a linear 5-20% sucrose gradient and centrifuged at 77,000 g for 16 h. Separation of the components was monitored by absorbance readings at 280 nm. The results obtained are shown in Figure 3.

Following the initial centrifugation, two distinct peaks, exhibiting different sedimentation characteristics,

Figure 2

Schematic Representation of Method Used to Separate HAd4 Soluble Components

HA - Hemagglutination Assay

CF - Complement-Fixation Assay

HE - Hemagglutination-Enhancement Assay

HEC - Hemagglutination-Enhancement Antibody Consumption Assay

HIC - Hemagglutination-Inhibition Antibody Consumption Assay

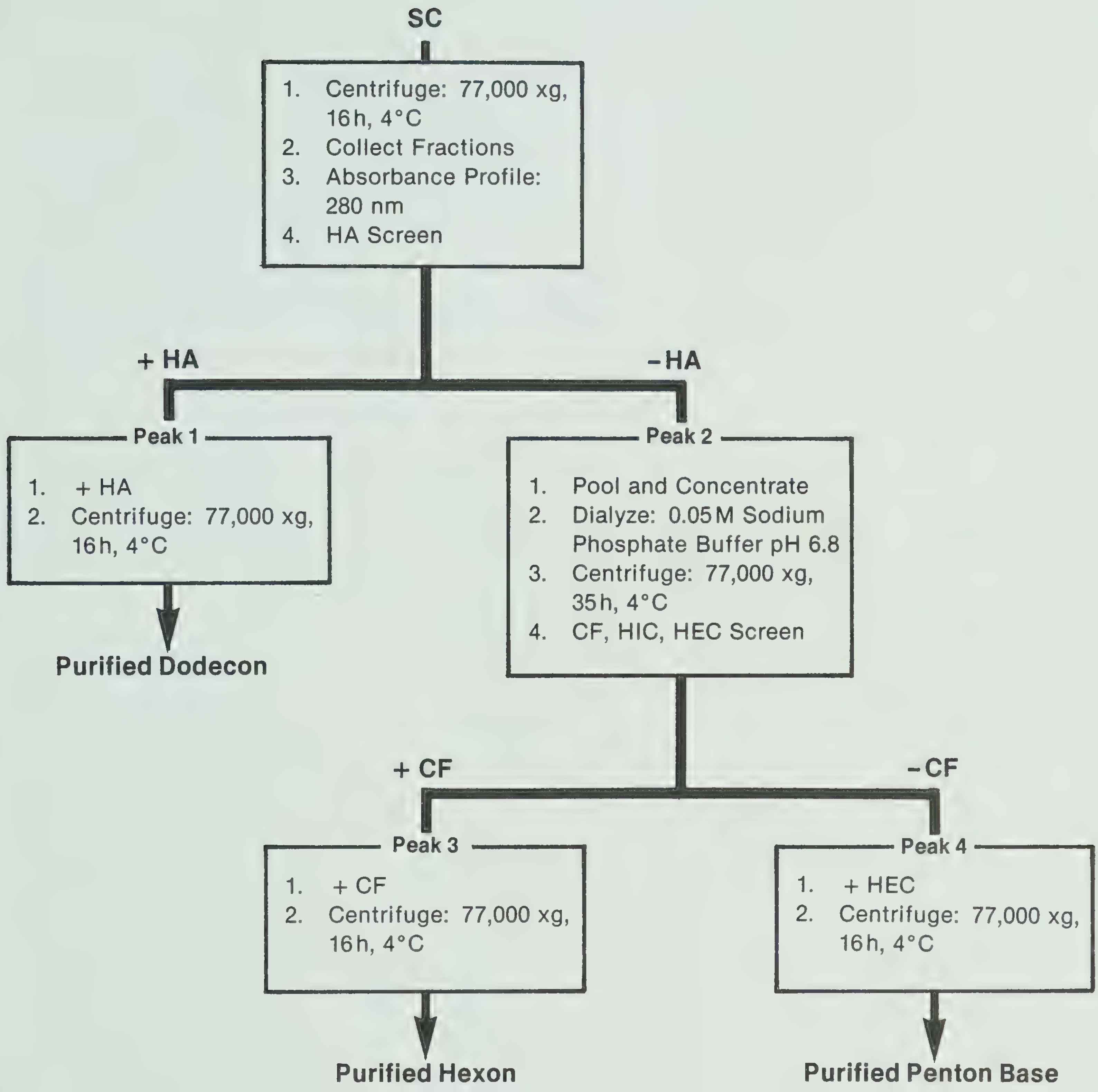
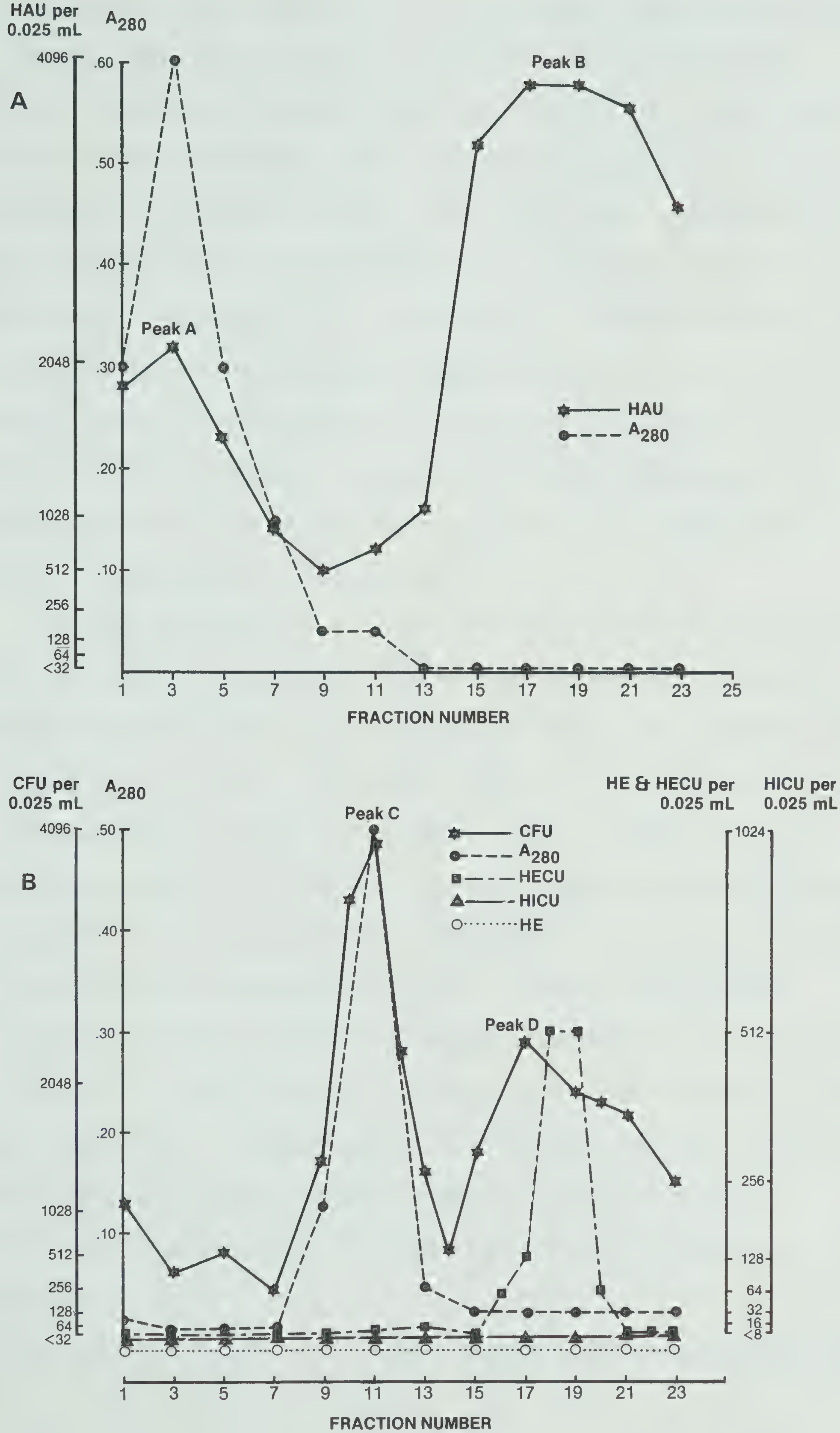

SC
1. Centrifuge: 77,000 xg, 16h, 4°C
2. Collect Fractions
3. Absorbance Profile: 280 nm
4. HA Screen
+ HA
- HA
Peak 1
1. + HA
2. Centrifuge: 77,000 xg, 16h, 4°C
Purified Dodecon
Peak 2
1. Pool and Concentrate
2. Dialyze: 0.05M Sodium Phosphate Buffer pH 6.8
3. Centrifuge: 77,000 xg, 35h, 4°C
4. CF, HIC, HEC Screen
+ CF
- CF
Peak 3
1. + CF
2. Centrifuge: 77,000 xg, 16h, 4°C
Purified Hexon
Peak 4
1. + HEC
2. Centrifuge: 77,000 xg, 16h, 4°C
Purified Penton Base

Figure 3

Separation of HAd4 Soluble Components by Rate Zonal Centrifugation as Determined by Serological Techniques and Absorbance Profile at 280 nm.

The SC material was separated as described in Methods and Materials.

A	-	Initial centrifugation, 77,000 g, 16 h, 4°C.
B	-	Centrifugation of Peak B, 77,000 g, 35 h, 4°C.
HAU	-	Hemagglutination units
HECU	-	Hemagglutination-Enhancement Antibody Consumption Units
HICU	-	Hemagglutination-Inhibition Antibody Consumption Units
HEU	-	Hemagglutination-Enhancement Units
CFU	-	Complement-Fixation Units
A280	-	Absorbance at 280 nm


HAU per 0.025 mL
A280
A
Peak A
Peak B
HAU
A280
FRACTION NUMBER
CFU per 0.025 mL
B
Peak C
Peak D
CFU
HECU
HICU
HE
HE & HECU per 0.025 mL
HICU per 0.025 mL

were distinguished (Figure 3A). The faster sedimenting peak was consistently recovered in the first 3-8 fractions, whereas the slower sedimenting peak exhibited a much wider profile extending from fractions 12-15 onwards to the top of the gradient. Hemagglutination activity (HA) was detected only in the faster sedimenting peak. Since the dodecon, a symmetrical aggregate of 12 pentons, is the only HAd4 soluble component expressing complete HA activity, this finding identified the peak as the dodecon population. The lack of HA in the slower sedimenting peak suggested that a complete separation of the dodecons from the remaining soluble components had occurred.

Further separation of the remaining soluble components was achieved by subjecting the slower sedimenting peak to recentrifugation. The centrifugation time was extended from 16 to 35 h to allow for maximum separation. Figure 3B shows the presence of two distinct peaks, one following closely behind the other. The faster sedimenting peak was recovered in the middle of the gradient and peak fractions were shown to contain hexon components by a CF assay, using HAd6 group-specific antiserum. The slower sedimenting peak did not exhibit a significant CF titre. The peak, however, did yield a positive hemagglutination-enhancement-consumption (HEC) reaction with subgroup specific antiserum, thus confirming the presence of penton base in the peak fractions.

Free penton and free fiber components were not detected

in any of the sucrose gradients examined, either by absorbance readings or by the presence of incomplete hemagglutinins as determined by the hemagglutination-enhancement (HE) reaction using HAd6 antiserum. The inability to isolate free penton suggested that the majority of the penton components were present in the stock material as dodecon and not as free pentons. The inability to detect the fibre component in any fraction was thought to be due to the presence of very low amounts of fibre, amounts insufficient to be detected by the methods employed.

C. IDENTIFICATION AND PURITY ESTIMATION OF ISOLATED SOLUBLE COMPONENTS

a) Two-Dimensional Immunoelectrophoresis

The purity of the separated soluble components as prepared by rate zonal centrifugation was determined by two-dimensional immunoelectrophoresis (2-D). This method, as described by Axelsen, Bock and Kroll (1973) allows for the identification of individual components on the basis of electrophoretic mobility. Each isolated soluble component was analyzed for its 2-D profile using antiserum prepared against disrupted, whole HAd4 virions. The results are shown in Figure 4. A single peak was observed for each structural component. Comparisons of the electrophoretic mobility of

Figure 4

Two-Dimensional Immunoelectrophoresis Profile of Individual HAd4 Soluble Components

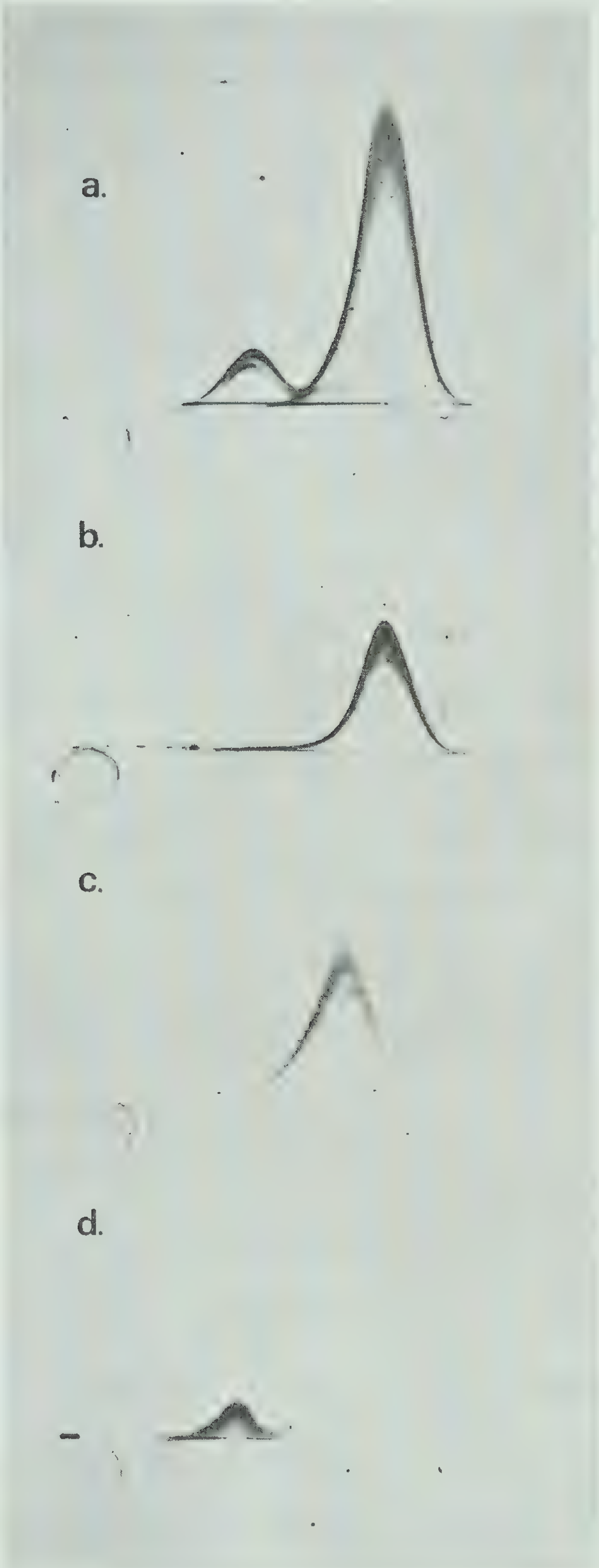
Twenty to forty uL of HAd4 SC stock material and separated soluble components were applied to 4 mm diameter wells at a protein concentration of 20-60 ug/mL. Fifty uL of antiserum directed against whole disrupted virions (final concentration, 0.6 uL/cm^2) was added to the agarose gel in the second dimension. Two-dimensional immunoelectrophoresis was carried out as described in Methods and Materials.

A. HAd4 SC stock

B. Hexon (peak C)

C. Penton Base (peak D)

D. Dodecon (peak A)


a.
b.
c.
d.

the individual components showed each to be unique and thereby identified each sample as a distinct and separate component. The hexon population exhibited the highest mobility and was followed closely by the penton base. The dodecon population, i.e. pentons, migrated the least from the origin. Each of the components was found to exhibit a similar mobility to one of the proposed component peaks from the HAd4 SC 2-D profile (refer to section D).

b) PAGE ANALYSIS

Final identification of the isolated soluble components was determined by SDS-PAGE analysis and is shown in Figure 5. The molecular weight of the individual components was calculated as described in Methods and Materials. The molecular weight (MW) of the hexon and penton (dodecon) components were calculated at 110,000 and 73,000, respectively (Figure 5, tracts E and F). The calculated MW of these components were found to be in agreement with those reported by Marusyk and Cummings (1978) and Wadell (1979). In addition, the presence of a single polypeptide band indicated that the hexon and penton preparations were relatively pure. In contrast to this finding, the isolated penton base preparation was found to be composed of a number of different polypeptides (Figure 5, tract D). Three major bands, with corresponding molecular weights of 60,000, 54,000 and 25,000 were observed. The latter two polypeptides were identified as the core protein, polypeptide V and the hexon-associated core protein, polypeptide VI of the HAd4

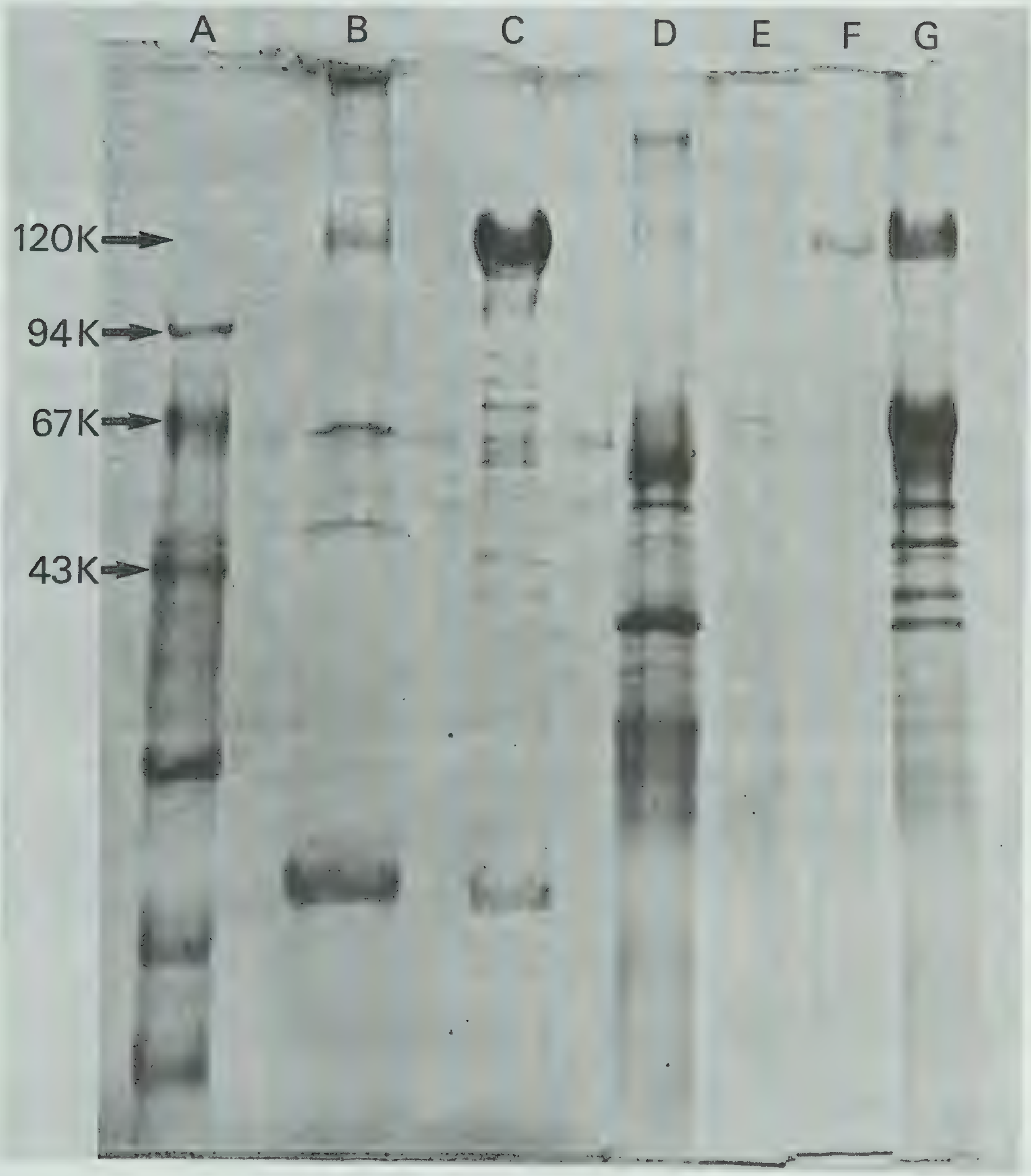
Figure 5

PAGE Analysis of Separated HAd4 Soluble Components

Samples were prepared as described in Methods and Materials and electrophoresed at 30 mA for 220 min.

A. Markers
B. HAd2
C. HAd4
D. Penton Base
E. Penton (Dodecon)
F. Hexon
G. HAd4 SC

Components were isolated from HAd4 SC material by rate zonal centrifugation.


A
B
C
D
E
F
G
120K
94K
67K
43K

virion.

The accepted MW of the penton base is estimated to be between 66,000-74,000 (Marusyk and Cumming, 1978; Wadell, 1979). No polypeptide band corresponding to that MW was observed by PAGE analysis. The inability to correlate the results of the two-dimensional immunoelectrophoresis profile with that of PAGE analysis, using identical penton base preparations, is not clear. It is possible that since the former technique is much more sensitive than that of the PAGE, the sample contained an insufficient amount of the penton base to be detected by PAGE analysis.

The nature of the 60,000 polypeptide band is not known, nor is the nature of the remaining polypeptide bands present in Figure 5, tract D. It is thought that these polypeptides may represent cellular contaminants.

D. TWO-DIMENSIONAL IMMUNOELECTROPHORESIS PROFILE OF HAd4 SOLUBLE COMPONENTS

a) Standardization

Two-dimensional immunoelectrophoresis can be used for the identification and quantitation of individual samples. Previous studies have reported its use for the characterization of adenovirus soluble components (Martin, Warocquier, and Boulanger, 1975). With this technique, identification and quantitation are performed in terms of a

reference antigen, i.e. a sample of known composition, and by means of a reference antiserum. Positive identity of any two antigens is indicated by identical electrophoretic mobility within a standardized system. In addition, the quantitation of the unknown antigen can be determined by the change in peak area resulting from the addition of the unknown antigen to that of the reference antigen (Axelsen et al., 1973).

In order to identify the individual components within HAd4 SC material, attempts were made to develop and standardize a 2-D profile of HAd4 SC material which exhibited good peak height with high resolution and separation of the viral soluble components. By varying the antiserum concentrations and electrophoresis times, a standard system was developed using Tris-glycine buffer, pH 8.3. The procedure and conditions used are as described in Methods and Materials.

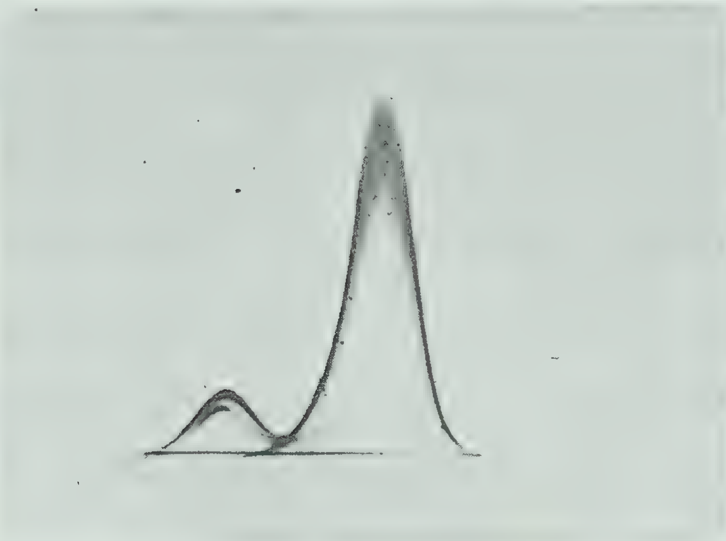
Under these conditions a specific pattern was observed in the 2-D profile of the HAd4 SC material. Repeated trials using different SC preparations resulted in similar profiles. Four potential peaks, possibly representing individual soluble components, were observed. A fifth minor precipitin line was also observed, however, this line was often masked by that of the others. The proposed peaks are shown in Figure 6 and have been referred to as 1, 2, 3, 4, and 5.

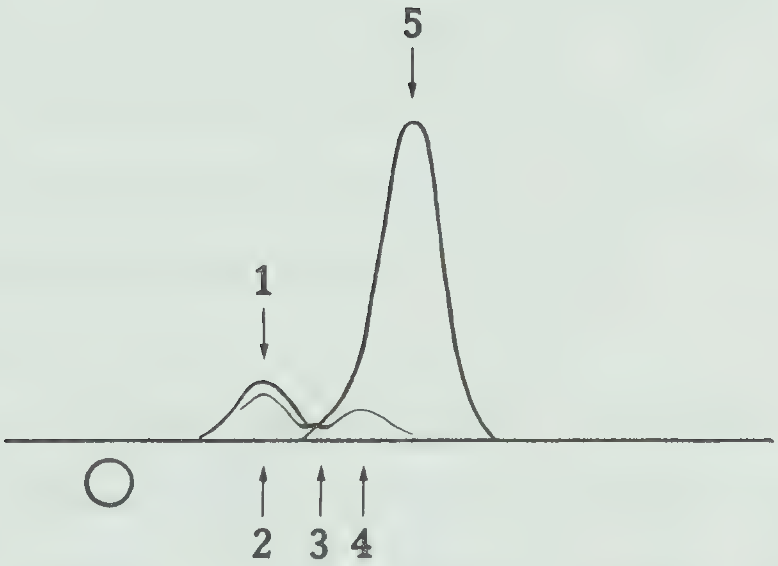
b) Identification of HAd4 2-D Profile Peaks

Figure 6

Two-Dimensional Immunoelectrophoresis Profile of HAd4 Soluble Components

HAd4 SC material was analyzed for its two-dimensional immunoelectrophoresis profile under the conditions described in Methods and Materials. Arrows indicate the proposed peaks.




5
1
2
3
4

Identification of the HAd4 2-D soluble component profile was determined by the use of an enhancing technique, as described by Axelsen et al. (1973). In this technique positive identity of precipitin peaks is determined on the basis of identical electrophoretic mobility of added antigen to the reference antigen and increased peak area. The 2-D profile of a standard amount of HAd4 SC material was used as a reference sample to which all other profiles were compared. Each purified soluble component was added separately to a standard amount of the reference sample after which the resulting 2-D profile was compared against that of the reference sample alone. The addition of purified hexon to the reference sample resulted in a greater than two-fold increase in the area of peak 5 compared to that of the reference profile. No increase in any of the other peaks was evident nor were any lines of identity observed. With the addition of purified dodecon to the reference sample an increase in both peaks 1 and 2 resulted. In comparison to the reference profile, peak 1 exhibited a 1.7-fold increase in area over that of the standard. The extent of the increase in peak 2 could not be determined due to an insufficient amount of the antigen in the reference profile. However, the finding that a sufficient amount was present to estimate area following dodecon addition indicated a direct relationship between the two. When a similar addition of purified penton base to the reference sample was examined, an increase in the area of peak 4 alone was observed. As was

the case previously with peak 2, the extent of this increase could not be determined due to the insufficient amount of antigen present in the reference profile. An identity reaction between peak 1 and 4 was evident.

In addition to the enhancement of the penton base peak, the 2-D profile permitted a more pronounced view of precipitin line 3 which was often masked by the other peaks. An identity reaction between this peak and peak 2 was evident.

The 2-D profiles of the above enhancement samples and their respective references are shown in Figure 7 (A,B and C). A tabulation of the peak areas and the relative changes in area is also given.

As a result of the above enhancement studies and the previous observation of the specific electrophoretic mobility of each individual soluble component (Figure 4), identification of the proposed peaks in the 2-D profile of the HAd4 SC material was possible. On the basis of these results, peaks 1, 2, 4, and 5 have been identified as the separate penton base and fiber structural components of the complete penton, the free penton base and the hexon component, respectively. Precipitin line 3 has been tentatively identified as the fiber component. The minimum amount of free fiber present in the SC material did not permit further investigation into the nature of peak 3. A revised two-dimensional profile of the HAd4 SC material is shown in Figure 8.

Figure 7A

Identification of Two-Dimensional Immunoelectrophoresis HAd4 Soluble Component Profile Peaks as Determined by the Enhancement Technique

The two-dimensional profile of a reference sample of HAd4 SC was compared to a similar sample to which a purified soluble component had been added. In each case, a standard concentration of the HAd4 SC reference sample was applied to both wells, although the original concentrations varied depending on the soluble component being examined. Tabulation of the peak area was calculated as the area enclosed in the precipitate and expressed as height x (width at half height). Those peaks marked with an asterisk represent the reference peaks to which the standard profile is identical.

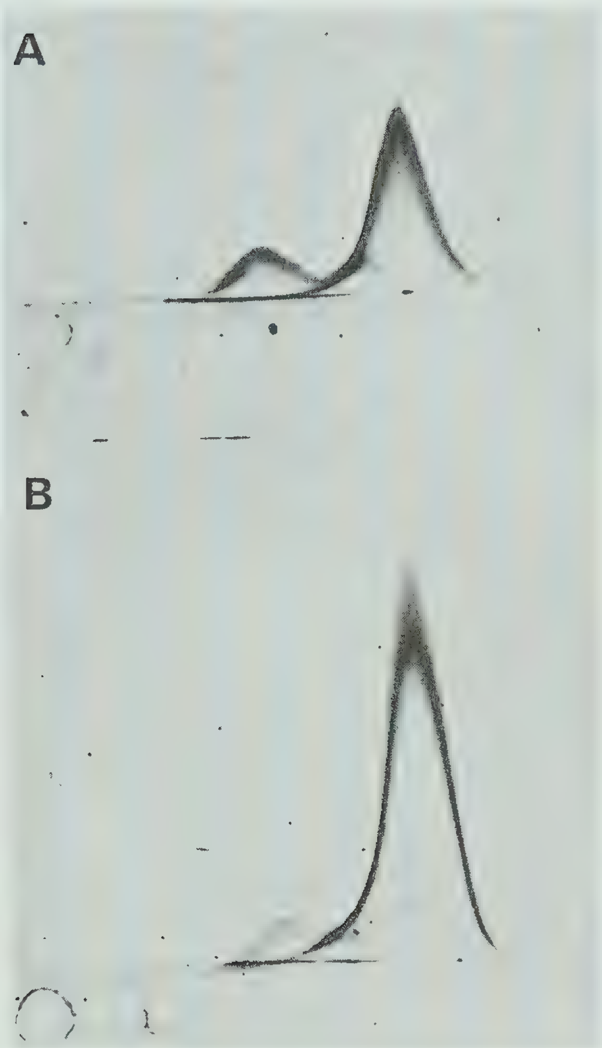
I. Enhancement of Hexon Component

A. Reference Profile

Load applied to well: 3 uL ; protein concentration, 0.62 ug/uL.

B. Hexon Enhancement Profile

Load applied to well: 25 uL; protein concentration, 0.06 ug/uL plus 3 uL HAd4 SC, as in A.



Sample	Peak Area (mm²)	
	*Peak 1	Peak 5
HAd 4 SC	43.2	128.7
HAd 4 SC + Hexon	43.2	308.0
Relative Change in Peak Area	0.0	+179.3
Relative Ratio of Peaks	1:1	1:7

Figure 7B

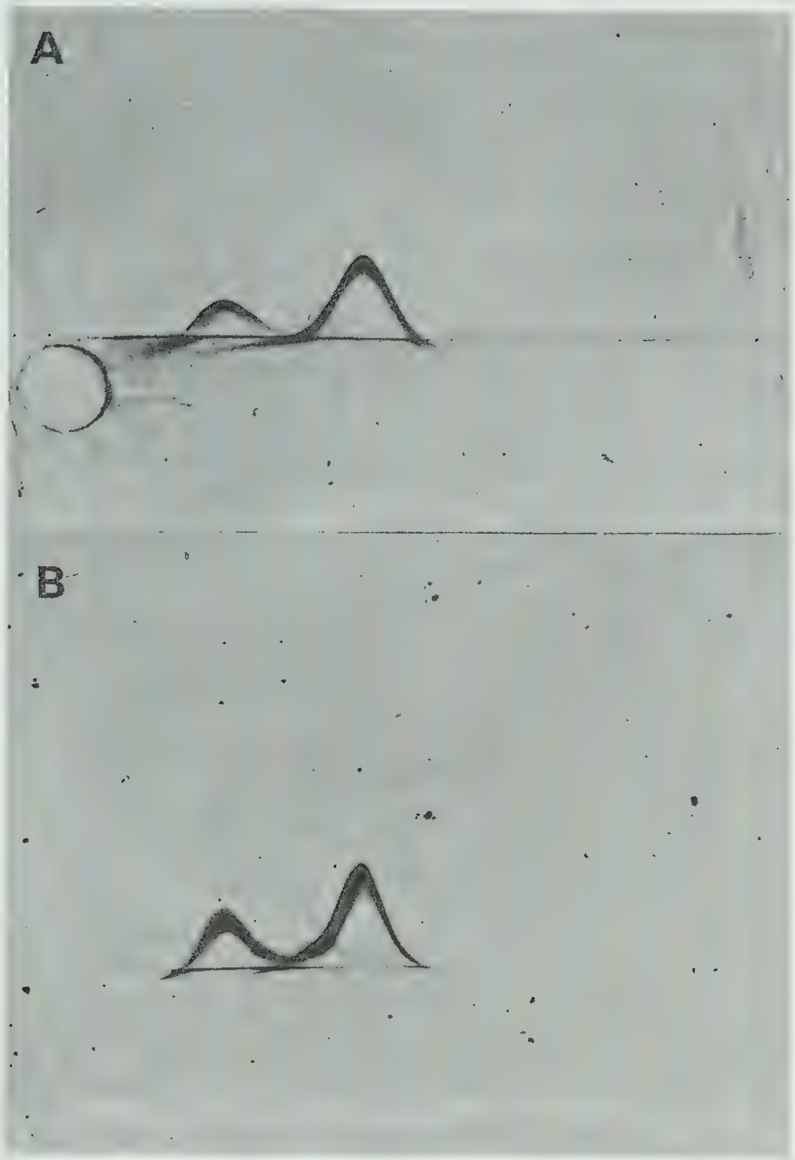
II.Enhancement of Complete Penton

A. Reference Profile

Load applied to well: 15 uL; protein concentration, 0.13 ug/uL

B. Complete Penton Enhancement Profile

Load applied to well: 20 uL ;protein concentration, 0.04 ug/uL plus 15 uL HAd4 SC, as in A.



Sample	Peak Area (mm²)		
	Peak 1	Peak 2	*Peak 5
HAd 4 SC	24.7	—	60.5
HAd 4 SC + Dodecon	39.6	28	57.2
Relative Change in Peak Area	+14.9	⩽ +28	−3.3
Relative Ratio of Peaks	1:1.7	1:>1	1:0.95

Figure 7C

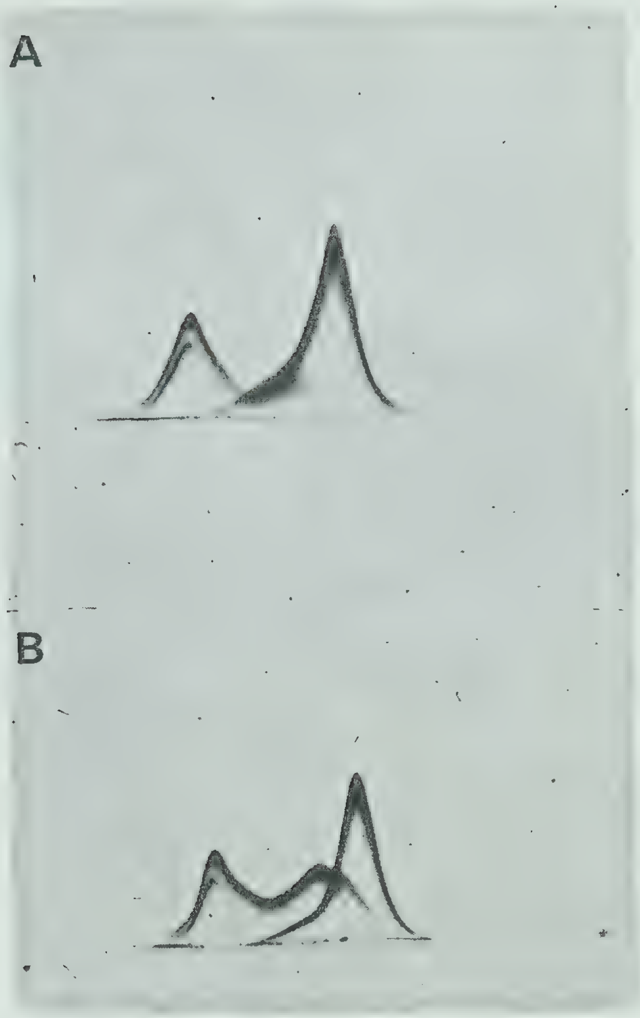
III. Enhancement of Penton Base

A. Reference Profile

Load applied to well: 15 uL; protein concentration, 0.13 ug/uL

B. Penton Base Enhancement Profile

Load applied to well: 7 uL; protein concentration, 0.07 ug/uL plus 15 uL HAd4 SC, as in A.



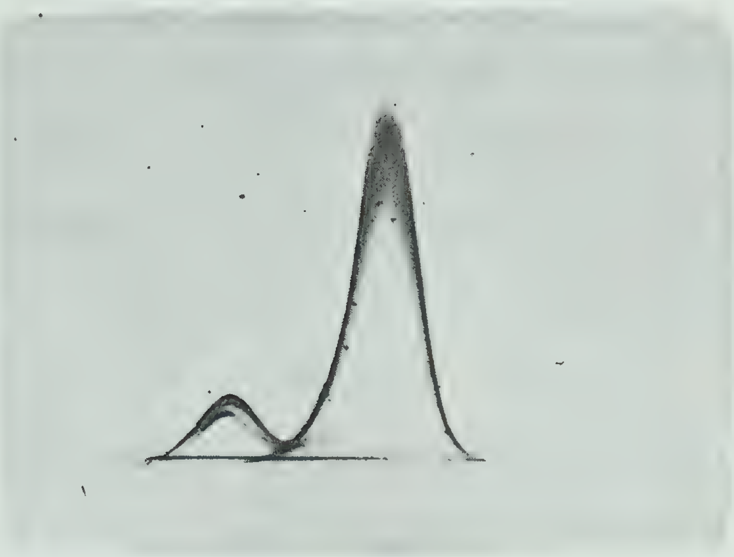
Sample	Peak Area (mm²)		
	*Peak 1	Peak 4	*Peak 5
HAd 4 SC	100	—	60.5
HAd 4 SC + Penton Base	89	66.3	55.0
Relative Change in Peak Area	−11	⩽ + 66.3	−4.5
Relative Ratio of Peaks	1:0.9	1:>1	1:0.9

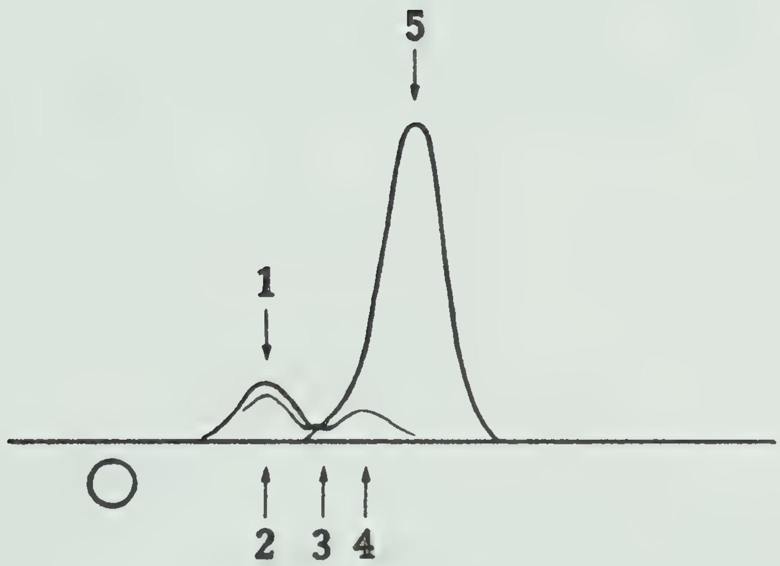
Figure 8

Two-Dimensional Immunoelectrophoresis Profile of HAd4 Soluble Components

On the basis of the enhancement studies and the specific electrophoretic mobilities of the individual soluble components, the proposed peaks have been identified as follows:

1. Penton base structural component of complete penton
2. Fiber structural component of complete penton
3. Free fiber
4. Free penton base
5. Hexon




5
1
2
3
4

E. GROWTH CHARACTERISTICS OF WT HAd4 AT 32, 37 and 39°C

The growth rate of WT HAd4 was examined at 32, 37 and 39°C. Examination of the growth cycle showed maximum yield of virus to be approximately the same at all three temperatures. An accelerated rate of multiplication, however, was observed at 37 and 39°C when compared to that at 32°C. Extracellular virus was detected at the former two temperatures, 37 and 39°C, by 16 h PI, with maximum yield being reached by 24 h. At 32°C, however, virus was not detectable until 24 h PI and maximum yield was reached at 48 h PI (Figure 9). No significant difference in the rate of multiplication between that at 37 and 39°C was observed.

Although the rate of multiplication of WT HAd4 varied at 32 and 39°C, no significant difference in the ability of the WT virus to grow at the permissive and non-permissive temperatures was observed. No restriction of growth at 39°C was apparent.

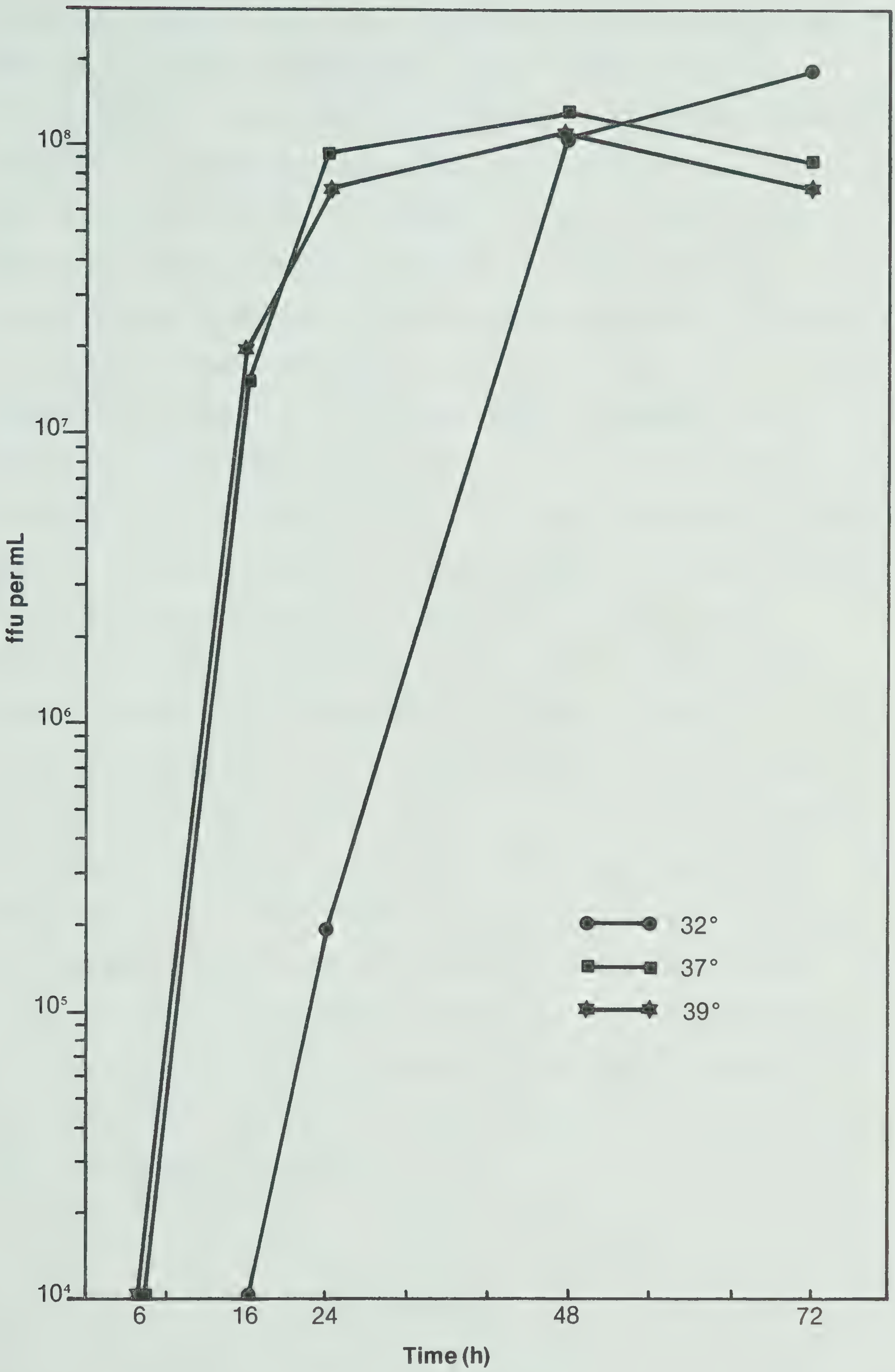
F. MUTANT ISOLATION

As previously indicated, five different mutagens have been employed in the attempt to isolate ts mutants of adenoviruses. Higher frequencies of mutant isolation following treatment with nitrous acid and hydroxylamine, however, have been reported (Williams et al., 1971; Ensinger et al., 1972; Begin and Weber, 1975). As a result of these

Figure 9

Growth Characteristics of WT HAd4 at 32, 37, and 39°C

HeLa cell monolayers in tissue culture tubes were infected with WT HAd4 at a MOI of 5 ffu/cell. After adsorption at 37°C for 1 h, the cells were washed once with PBS to remove excess virus after which maintenance media was added and the cells incubated at the respective temperatures. Cell cultures were harvested at the time intervals indicated and viral yield determined by the fluorescent-focus assay.



ffu per mL
10⁸
10⁷
10⁶
10⁵
10⁴
6
16
24
48
72
Time (h)
32°
37°
39°

findings, mutagenization of stocks of HAd4 was restricted to the use of these two mutagens. Both nitrous acid and hydroxylamine inactivated the HAd4 virus stock by single-hit kinetics, as demonstrated by the rate of HAd4 inactivation. The results are shown in Figures 10 and 11. Due to the plaquing inefficiency of the wild type virus, mutant screening was modified to involve microscopically locating individual plaques followed by screening for temperature-sensitivity, as described in Methods and Materials. Over 500 plaques grown from a virus stock previously treated with nitrous acid were examined in this manner. A single mutant, designated HAd4 ts1, was isolated from this population and its efficiency at producing infectious virus examined at 32 and 39°C. A 1000-fold greater capacity of the mutant to produce infectious virus at 32°C to that at 39°C was observed by the $TCID^{50}$ assay; the 32°C:39°C ratio being $10^4:10^1$. The reversion frequency of the mutant was not determined. Wild type HAd4 virus exhibited a 32°C:39°C infectivity ratio of approximately 1.0. Comparisons of the efficiency of the mutant and WT virus to produce infectious virions is given in Table 3.

Mutant isolation following hydroxylamine treatment was also examined. However, no mutants were isolated from over the 1000 plaques screened.

Figure 10

Nitrous Acid Inactivation of HAd4

WT HAd4 virus stock was mutagenized with nitrous acid as described in Methods and Materials. The reaction was stopped at various intervals by the addition of cold Tris-HCl buffer. Viral survival was calculated by the decrease in number of fluorescent-focus units with time compared to a reference control of HAd4 virus stock maintained under identical conditions.

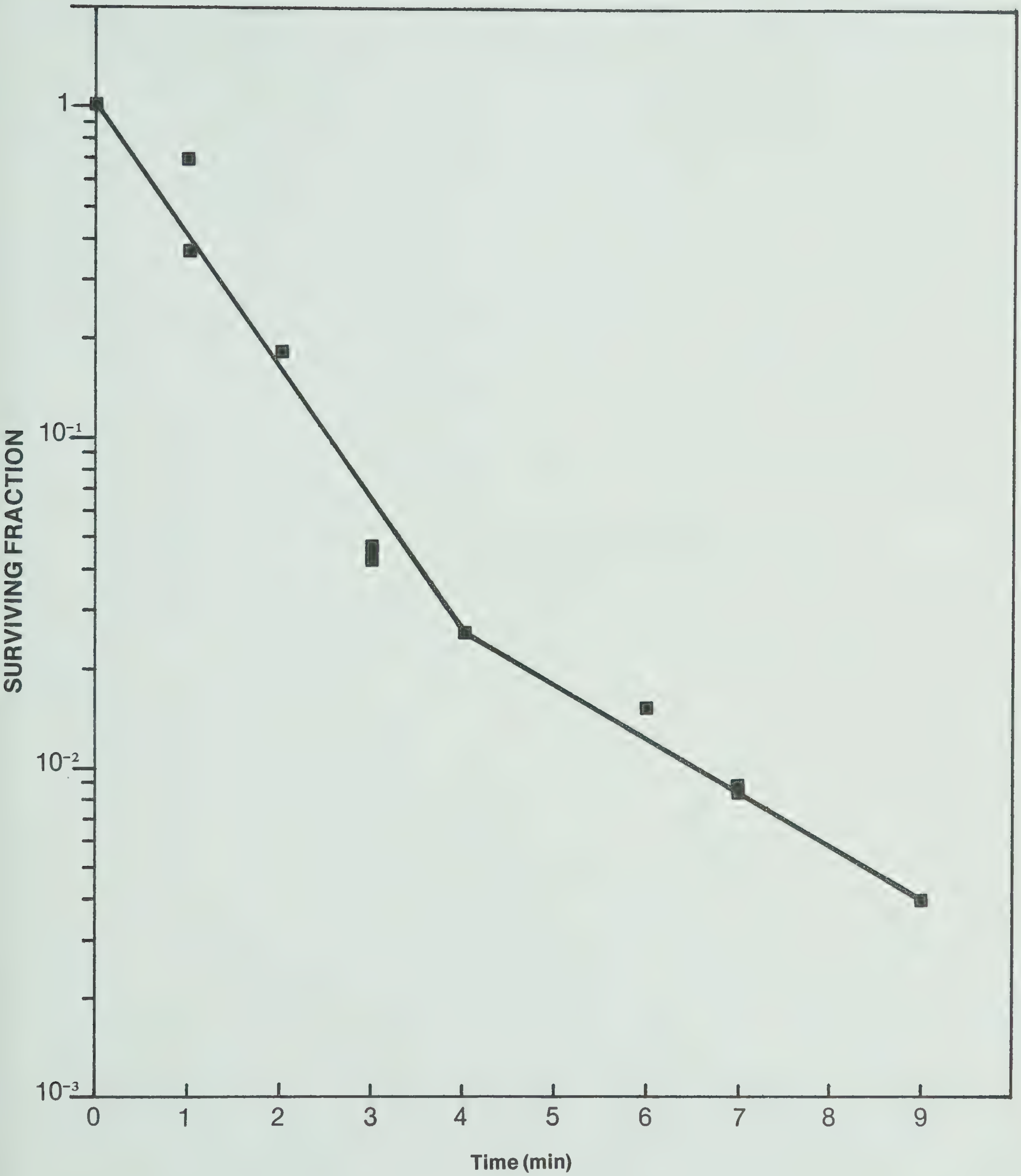

SURVIVING FRACTION
1
10⁻¹
10⁻²
10⁻³
0
1
2
3
4
5
6
7
8
9
Time (min)

Figure 11

Hydroxylamine Inactivation of HAd4

Hydroxylamine mutagenization was performed as described in Methods and Materials. Inactivation of WT HAd4 virus stock was calculated as outlined in Figure 10.

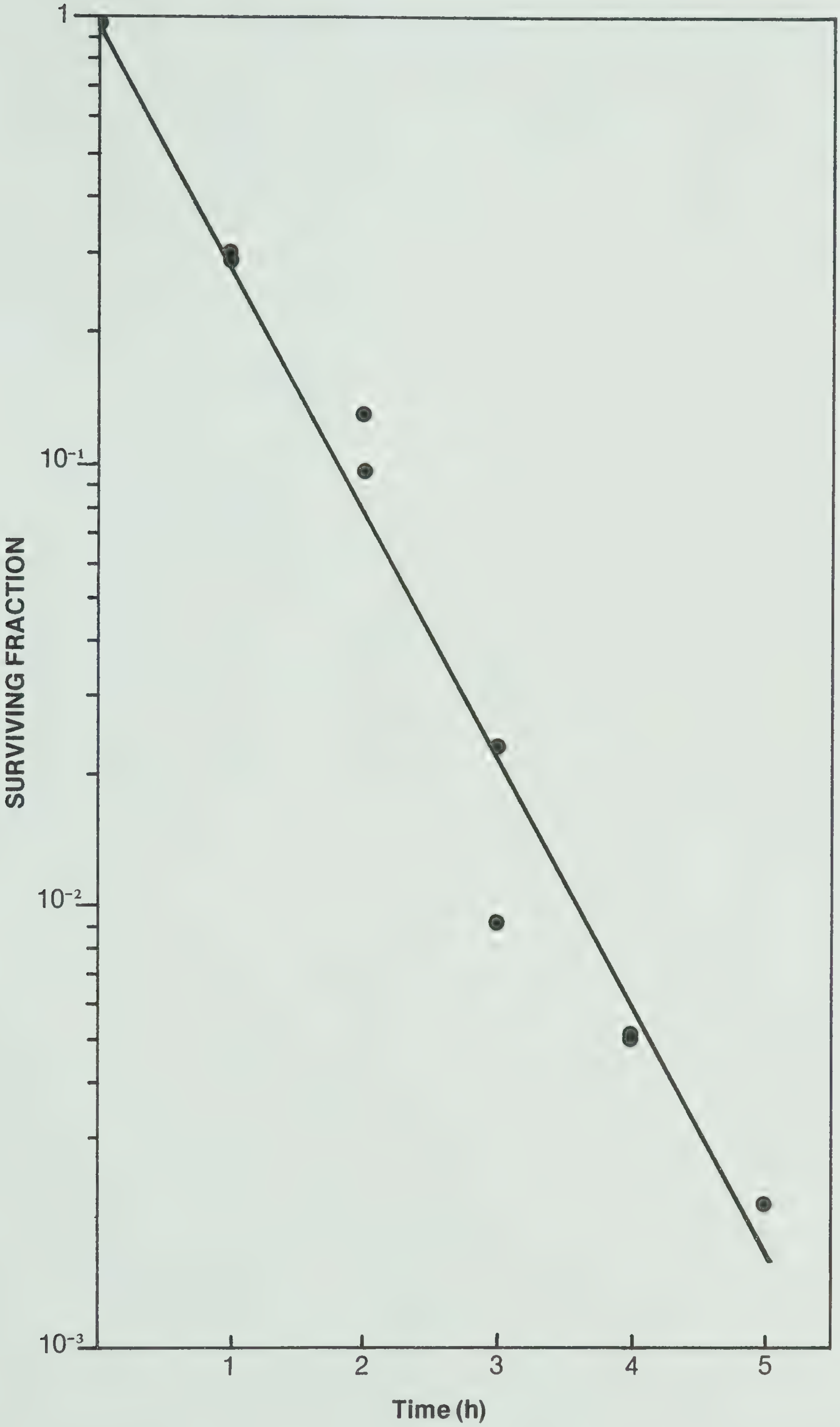

1
10⁻¹
10⁻²
10⁻³
SURVIVING FRACTION
1
2
3
4
5
Time (h)

Table 3

Efficiency of HAd4 ts1 Virion Production at Permissive and Non-Permissive Temperatures

Virus	Infectivity ($TCID_{50}$/0.1 mL)		Ratio of $TCID_{50}$ Infectivity (32°C/39°C)
	32°	39°	
Wild Type	**10^5**	**10^5**	**10^0**
HAd 4 ts 1	**10^4**	**10^1**	**10^3**

G. TWO-DIMENSIONAL CHARACTERIZATION OF HAd4 ts1

a) Two-Dimensional Profile of HAd4 ts1 at 32 and 39°C

The capacity of the HAd4 ts1 mutant to induce the synthesis of immunologically active soluble components was examined at both the permissive and non-permissive temperatures by two-dimensional immunoelectrophoresis. At the permissive temperature, normal production of the soluble components by the mutant was evident and the relative amounts of the soluble components produced were found to be similar to that produced by the wild type virus. The results are shown in Figure 12. A significant difference in the 2-D profile of the mutant compared to the wild type, however, was observed at the non-permissive temperature. Three of the structural components, the complete penton, penton base and fiber, were not detected. The respective profiles are illustrated in Figure 13. Only the hexon component showed normal production and this production was found to occur irrespective of the adsorption temperature employed. Occassionally, a slight shoulder was observed on the trailing edge of the hexon peak. Although no definite peak could be discerned, the location of the shoulder suggested that it may reflect miminum production of either penton base or fiber.

b) Effect of Temperature-Shift on the Expression of the Capsid Components as Determined by Two-Dimensional Immunoelectrophoresis.

The effect of temperature-shift on the presence of

Figure 12

Two-Dimensional Immunoelectrophoresis Profile of HAd4 ts1 at 32°C

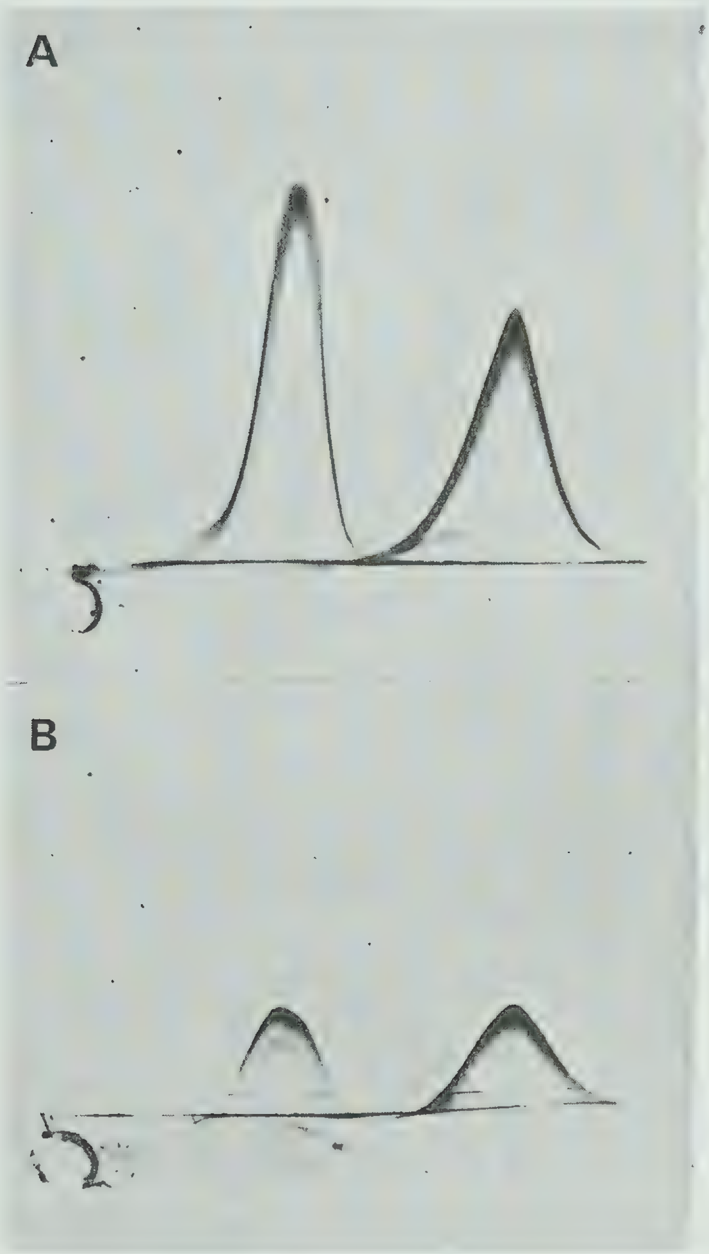
HeLa cells were infected with HAd4 ts1 at a MOI of 5 ffu/cell. The cells were incubated at 32°C for 72 h after which the cells were harvested and virus extracted as described in Methods and Materials. The resulting two-dimensional profile was then compared against that of reference HAd4 also grown at 32°C.

A. WT HAd4 Profile

Load applied to well: 15 uL cell extract

B. HAd4 ts1 Profile

Load applied to well: 25 uL cell extract



Sample	Peak Area (mm^2)		Relative Ratio of Peak Areas
	Peak 1	**Peak 5**	
HAd 4 ts 1	**86.4**	**93.6**	**1:1**
HAd 4	**249.6**	**222.7**	**1:1**

Figure 13

Two-Dimensional Immunoelectrophoresis Profile of HAd4 ts1 at 39°C

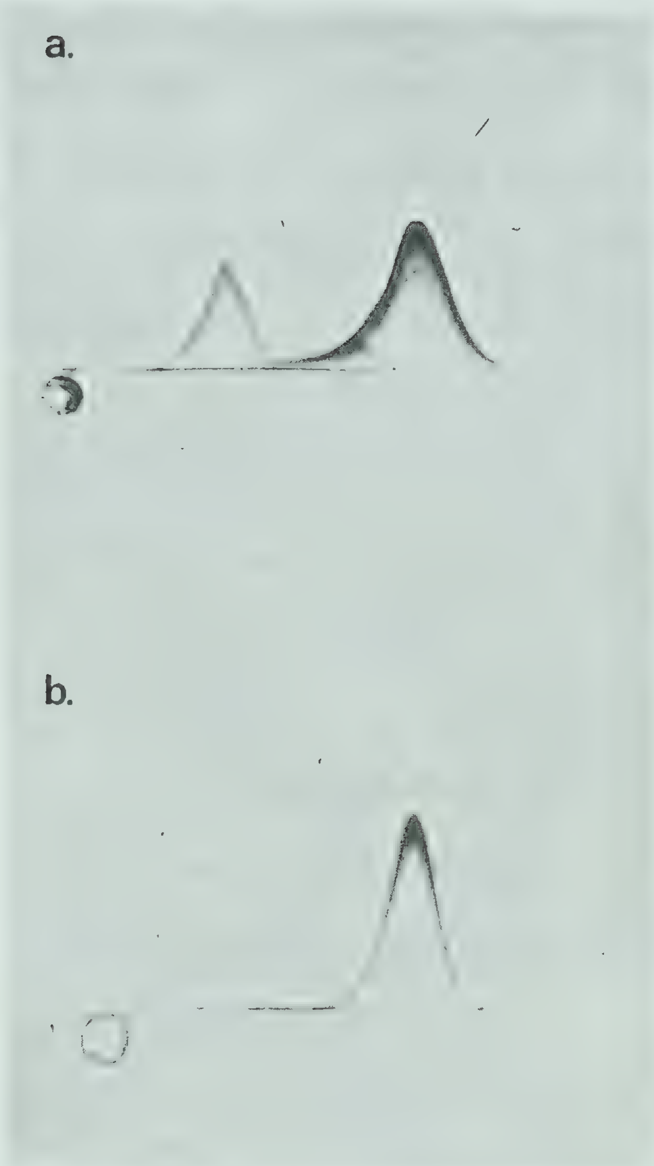
The experimental protocol followed in this study was as previously described in Figure 12, with the exception that incubation was carried out at 39°C. The reference profile used was of WT HAd4 grown at 39°C.

A. WT HAd4 Profile

Load applied to well: 20 uL of cell extract

B. HAd4 ts1 Profile

Load applied to well: 20 uL of cell extract


a.
b.

the complete penton, penton base and fiber was analyzed by examination of the 2-D profile of mutant-infected cell extracts. Since production of these components occurred only at the permissive temperature, three questions were raised: (1). Can the mutant grown at 39°C re-establish normal production of immunologically active soluble components at 32°C ?; (2). Can the defect expressed at 39°C be overcome by an initial incubation at 32°C ?; (3) Is the defect in the synthesis of the components or in the processing of the components ?

The 2-D profiles resulting from a shift-down experiment from 39 to 32°C for 48 h showed a reappearance of the missing components to occur as observed by the presence of immunologically active complete penton, penton base and fiber. Although the fiber peak was not distinct in the 2-D profile, its re-establishment was assumed on the basis of the presence of complete penton, which is composed of both penton base and fiber (Figure 14).

The effect of shift-up to the non-permissive temperature was also examined in order to determine whether the defect could be overcome by an initial incubation at the permissive temperature. One complete cycle of adenovirus replication is known to take approximately 16-24 h at 37°C with synthesis of the structural components occurring by 14 h postinfection (Philipson, Pettersson, and Lindberg, 1975). An initial incubation of 48 h at 32°C would ensure that the synthesis of the structural components as well as the

Figure 14

Effect of Temperature Shift-down on the Two-Dimensional Immunoelectrophoresis Profile of HAd4 ts1

HeLa cells were infected with HAd4 ts1 at a MOI of 5 ffu/cell. Adsorption of the mutant was carried out at 39°C. The cells were incubated for 36 h at the non-permissive temperature followed by a shift-down to the permissive temperature for a period of 72 h. The cells were then harvested and virus extracted as previously described. The resulting two-dimensional profile was compared against that of a mutant control at 39°C and a WT control at 37°C

A. HAd4 ts1 Profile after shift-down

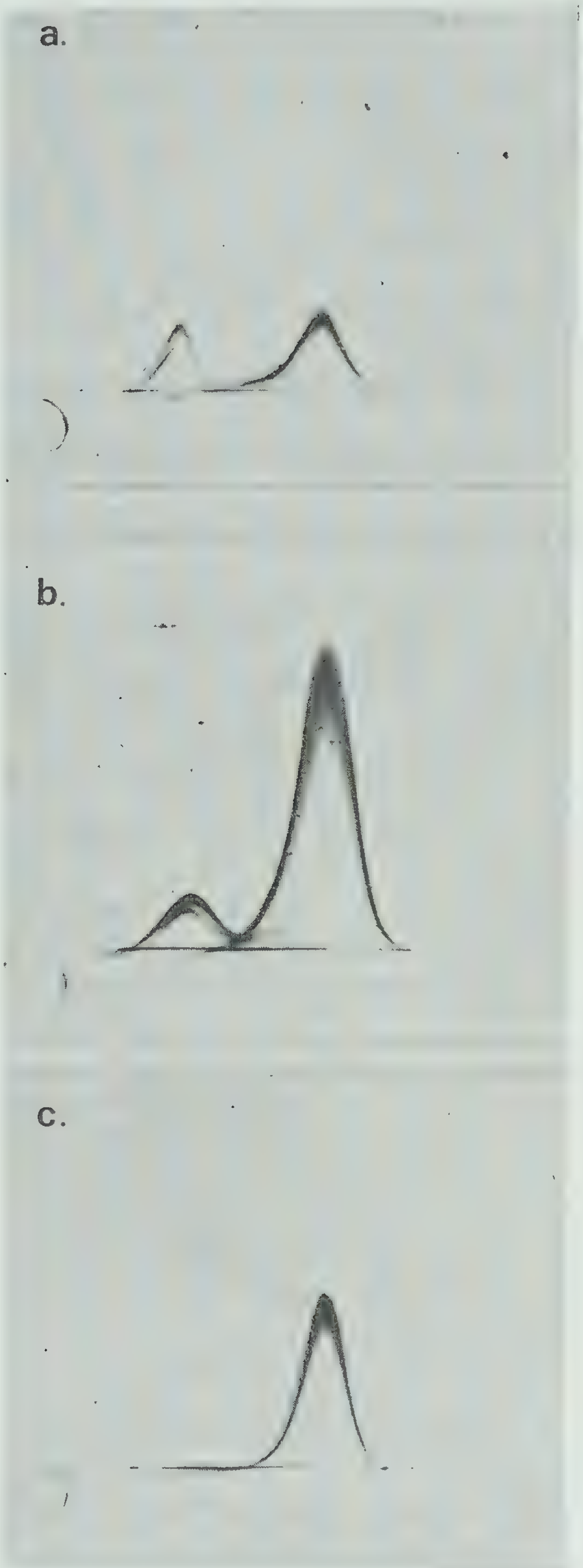
Load applied to well: 20 uL of cell extract

B. WT HAd4 Profile

Load applied to well: 15 uL of cell extract

C. HAd4 ts1 Profile at 39°C

Load applied to well: 20 uL of cell extract


a.
b.
c.

production of the remaining viral polypeptides had occurred. A full complement of the viral polypeptides necessary for replication would, therefore, be present. No reappearance of the components, following shift-up to 39°C, however, was observed. This indicated, therefore, that the defect could not be overcome by an initial incubation at 32°C and that the defect was expressed at the nonpermissive temperature.

The nature of the defect was further examined by the 2-D profiles of mutant-infected cell extracts following a shift-down to the permissive temperature for variable time periods (Figure 15). Immunologically active complete penton and penton base were observed within 8 h after shift-down. A further increase in these components was evident within 24 h. The reappearance of peak 4, that of the penton base, was particularly significant in view of the amount normally produced by the mutant at 32°C. A 1.8-fold increase over that of the mutant control was observed.

H. HEAT STABILITY

The structural integrity of the mutant compared to that of the WT virus was examined by determining the relative heat stability of the two virus populations at 39°C. Crude virus samples of both mutant stocks and WT virus were tested together in order to maintain uniform conditions. The results are shown in Figure 16.

Figure 15

Time-Sequence Analysis of the Effect of Temperature Shift-Down on the Two-Dimensional Immunoelectrophoresis Profile of HAd4 ts1

HeLa cells were infected with HAd4 ts1 at a MOI of 5 ffu/cell. Virus was adsorbed for 2 h at 32°C after which maintenance media was added and the cells incubated at the non-permissive temperature. At 24 h PI, the cells were shifted-down to 32°C for 8 and 24 h periods. Following incubation, the cells were harvested and virus extracted as described in Methods and Materials. The two-dimensional profile of the resulting cell extract was analyzed for the presence of complete penton, penton base and fiber. HAd4 ts1 controls at 32 and 39°C were included in the study. In these instances, the cells were harvested and virus extraced at 72 and 36 h PI, respectively.

A. HAd4 ts1 Profile at 32°C

Load applied to well: 30 uL cell extract

B. HAd4 ts1 Profile at 39°C

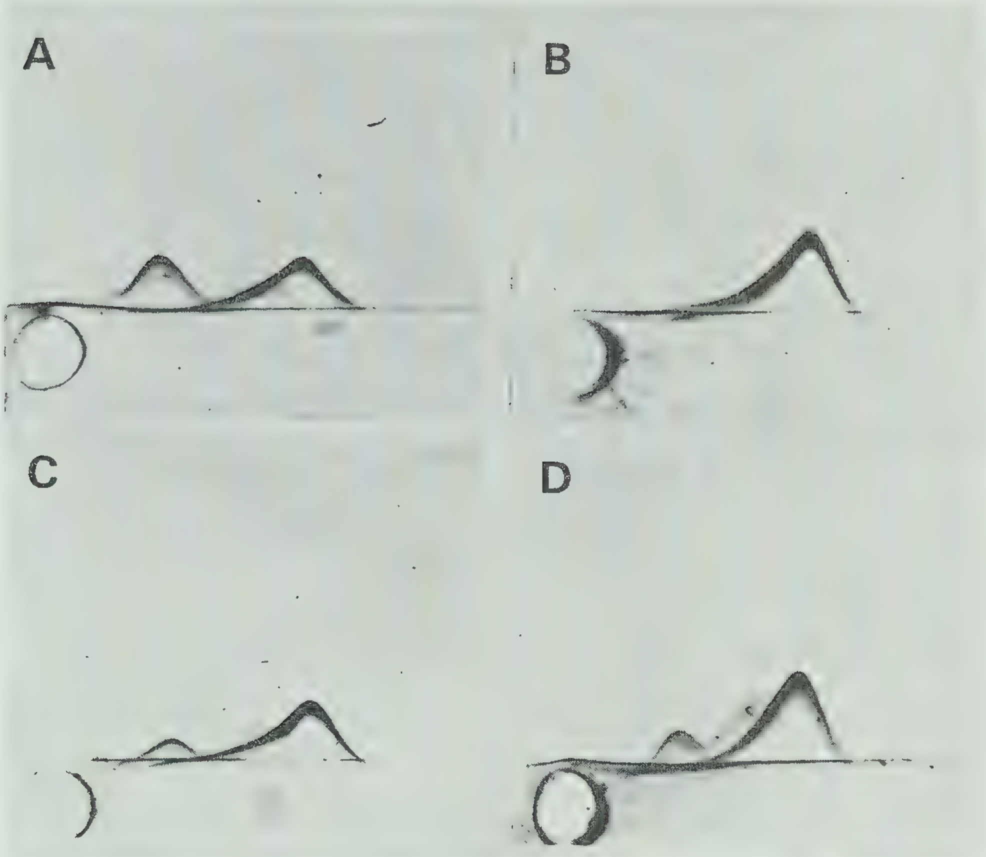
Load applied to well: 30 uL cell extract

C. HAd4 ts1 Profile: 8 h post shift-down

Load applied to well: 30 uL cell extract

D. HAd4 ts1 Profile: 24 h post shift-down

Load applied to well: 30 uL cell extract

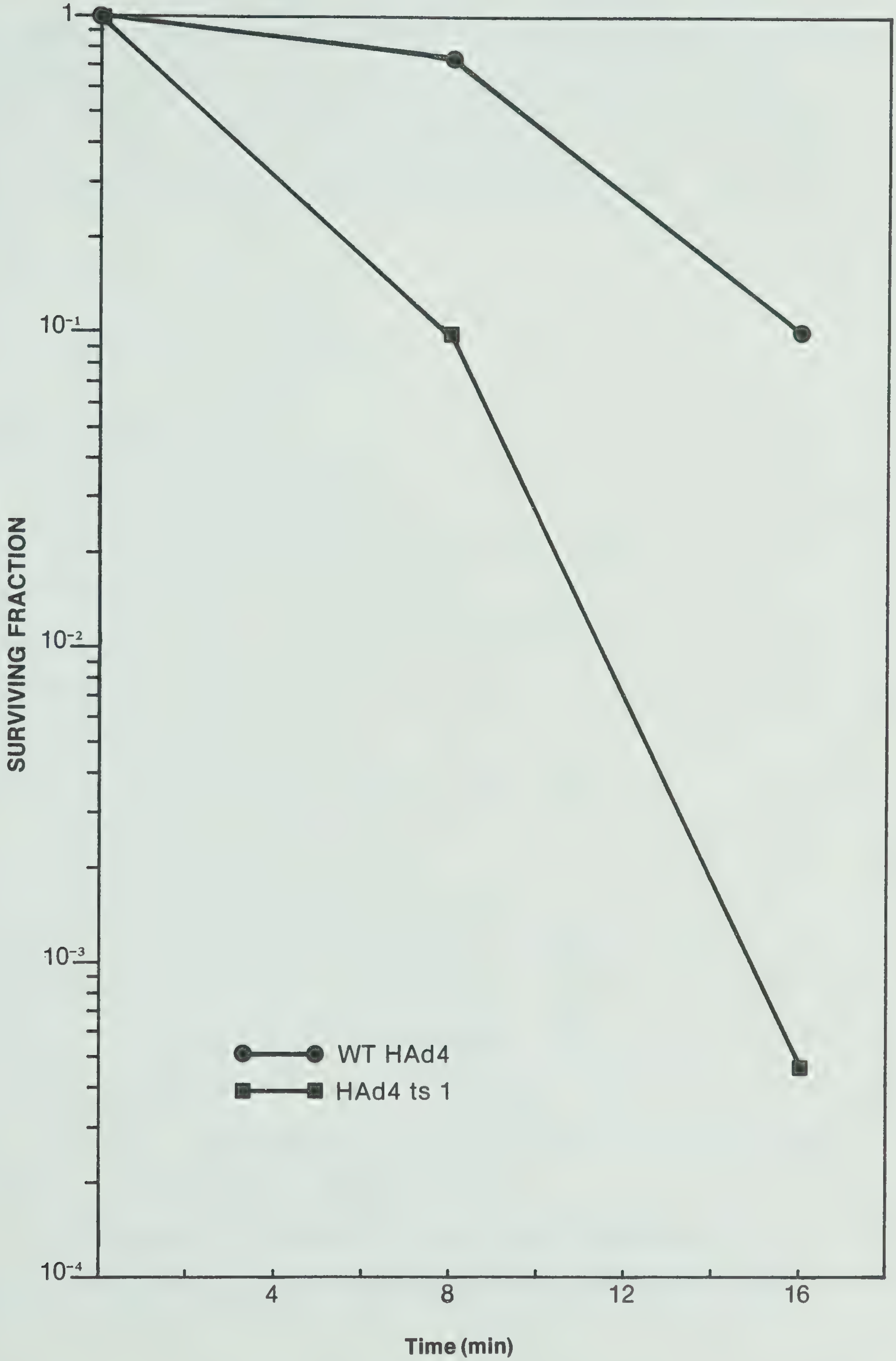


Sample	Peak Area (mm²)			Relative Ratio of Peaks 1, 3 and 5
	Peak 1	Peak 3	Peak 5	
ts 1:39°C	—	—	60.2	—
ts 1:32°C	33.6	16.5	36.4	0.9:0.5:1.0
ts 1:shift-down 8 hours	8.0	21.6	43.4	0.2:0.5:1.0
ts 1:shift-down 24 hours	18.0	53.2	62.4	0.3:0.9:1.0

Figure 16

Heat Inactivation of Virions of HAd4 ts1

Duplicate samples of 0.2 mL of crude HAd4 ts1 and WT HAd4 virus grown at 32 and 37°C, respectively, were immersed in a water bath at 50°C. Samples were harvested at the indicated times and cooled rapidly in an ice bath. Viral titer was determined by the fluorescent-focus assay.


1
10^{-1}
10^{-2}
10^{-3}
10^{-4}
SURVIVING FRACTION
4
8
12
16
Time (min)
WT HAd4
HAd4 ts 1

Following incubation at 50°C, the infectivity of the HAd4 ts1 mutant was found to be inactivated more rapidly than that of the WT virus. Only 10% of infectious mutant survived after 8 min of heating at 50°C. A 16 min heating period was required to reduce WT infectivity to a similar level.

I. DNA SYNTHESIS

The ability of the HAd4 ts1 mutant to synthesize DNA at both the permissive and non-permissive temperatures was examined by velocity sedimentation in alkaline sucrose gradients of newly synthesized (^3H)-thymidine labeled DNA extracted from infected cell cultures. The method used was as described by Weber et al. (1975). Details of the procedure are outlined in Methods and Materials.

In this series of experiments, location of the viral DNA peak was determined by comparing the sedimentation pattern from WT HAd4 infected cell cultures to that from uninfected cell controls. As shown in Figure 17, a peak, present in the middle of the gradient, was detected in the velocity sedimentation pattern obtained from the infected cell cultures. The absence of this peak in the uninfected controls identified it as viral DNA.

The velocity sedimentation pattern obtained with the HAd4 ts1 mutant was then compared at the permissive and

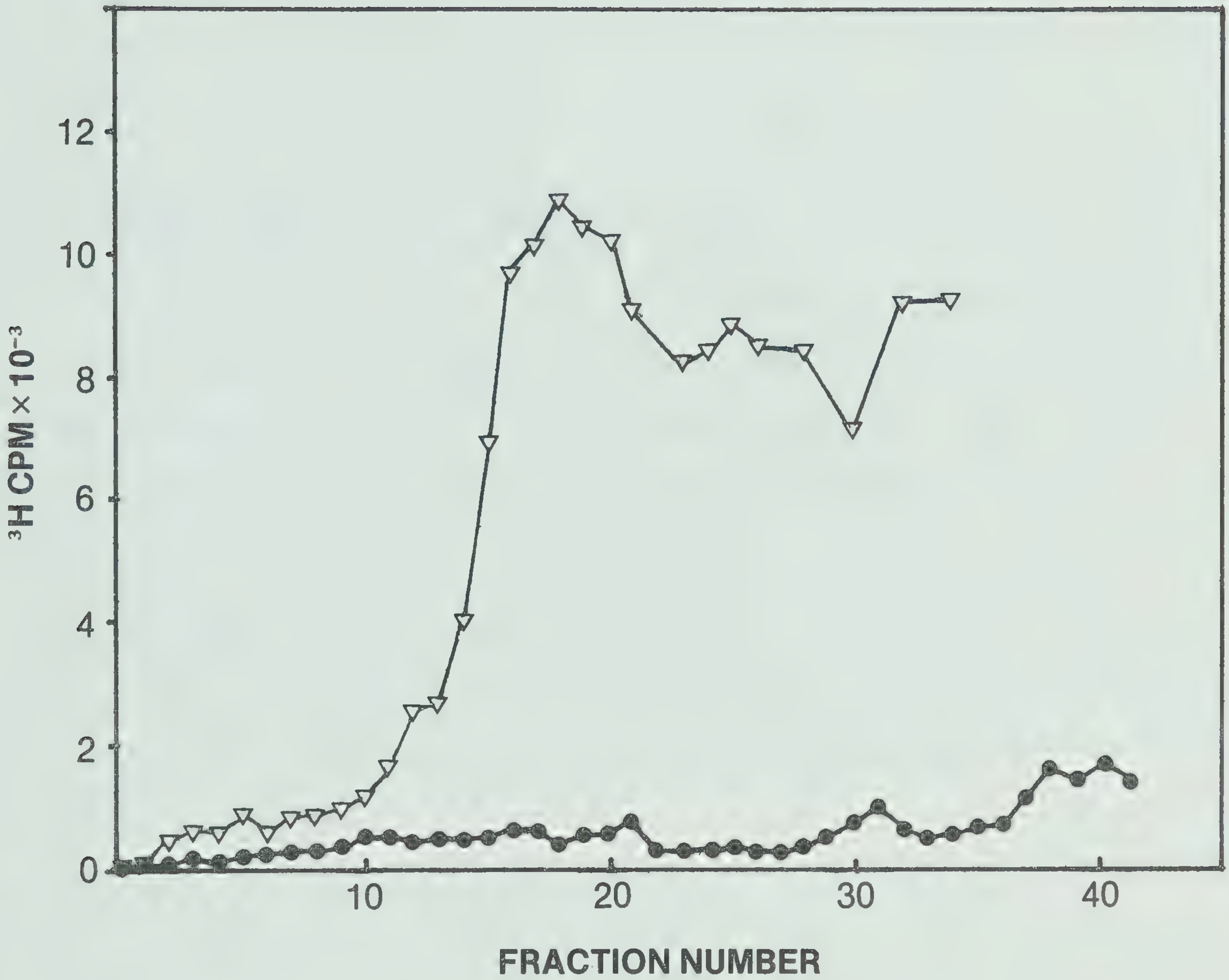
Figure 17

Comparison of the Velocity Sedimentation Profile of Viral and Whole Cell DNA Obtained from HAd4 Infected Cell Extracts and Uninfected Controls

HeLa cells were labelled with (^{3}H)-thymidine for 16 h at 20 h PI. The protocol followed for velocity sedimentation was as described in Methods and Materials.

●-● Velocity sedimentation profile of uninfected controls

▽-▽ Velocity sedimentation profile of HAd4 infected cell cultures


12
10
8
6
4
2
0
^{3}H CPM × 10^{-3}
10
20
30
40
FRACTION NUMBER

non-permissive temperatures. The results are shown in Figure 18. The presence of viral DNA synthesis was detected in mutant-infected cell cultures at both the permissive and restrictive temperatures. No significant difference in the level of DNA synthesis induced by the mutant to that of the WT was apparent.

J. VIRUS STABILITY IN CESIUM CHLORIDE

The ability of the HAd4 ts1 mutant to assemble virus at the non-permissive temperature was examined by analyzing for the presence of nucleic acid containing virion particles following equilibrium centrifugation in cesium chloride . Viral assembly was observed to occur at both the permissive and non-permissive temperatures. A 40% reduction in the ability of the mutant to produce virus at 39°C compared to that at 32°C was observed. The results are shown in Figure 19.

K. VIRUS ASSEMBLY

Virus assembly was studied by electron microscopy of cells infected with the HAd4 ts1 mutant at both the permissive and non-permissive temperatures, using wild type HAd4 infected cells grown at 37°C, as the control. A blind

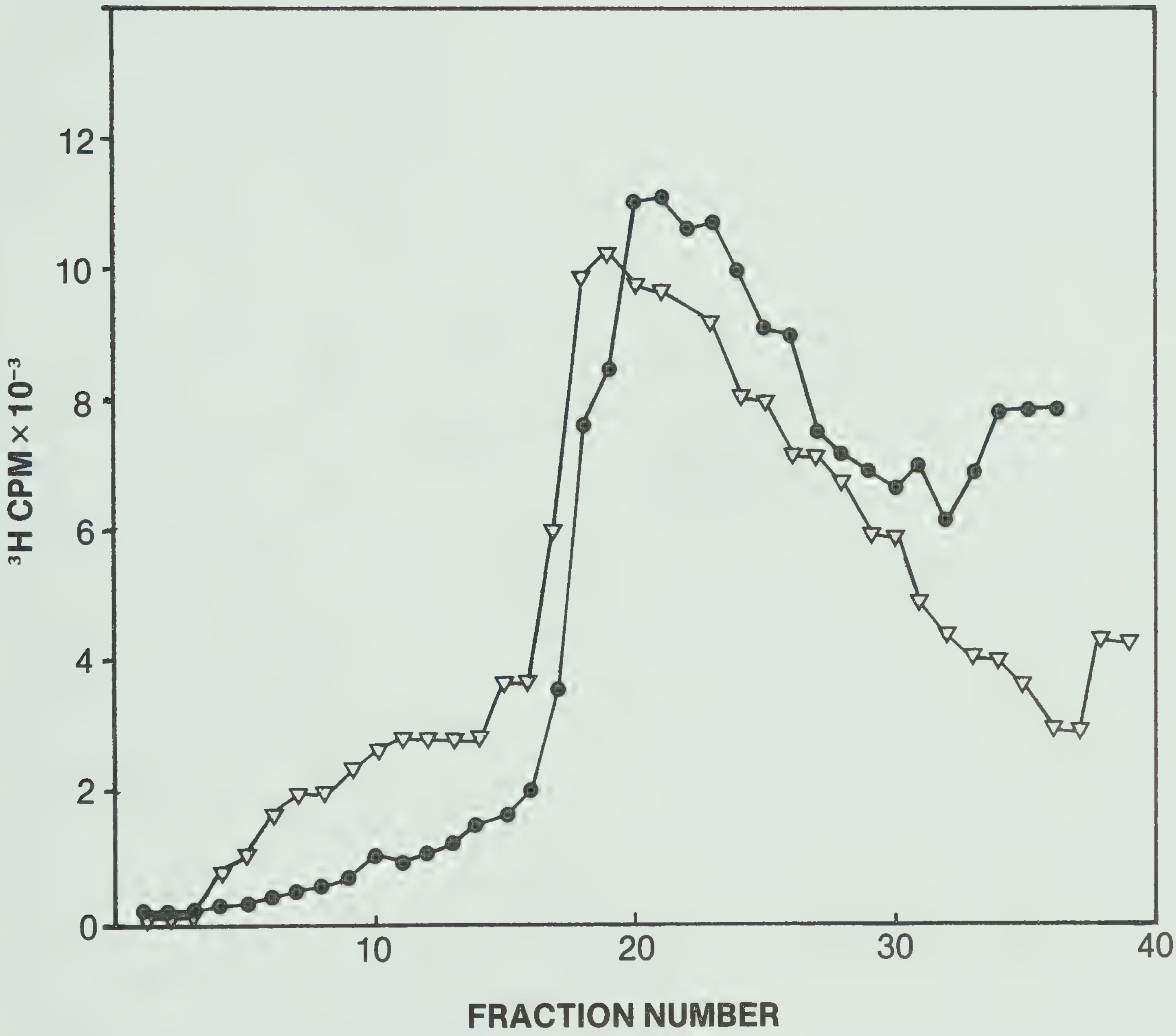
Figure 18

Velocity Sedimentation Profile of HAd4 ts1 at 32 and 39°C

HeLa cells were infected with HAd4 ts1 at a MOI of 10 ffu/cell. The cells were incubated at 39 and 32°C and labeled with 5 uCi/mL of (^{3}H)-thymidine at 20 and 40 h PI, respectively. Cells incubated at 39°C were harvested at 40 h PI while those incubated at 32°C were harvested at 65 h PI. The cells were analyzed for the production of viral DNA by alkaline sucrose gradient velocity sedimentation. Details of the procedure are as outlined in Methods and Materials.

▽-▽ (^{3}H)-thymidine counts/min incorporated at 32°C

●-● (^{3}H)-thymidine counts/min incorporated at 39°C



12
10
8
6
4
2
0
^3H CPM × 10^{-3}
10
20
30
40
FRACTION NUMBER

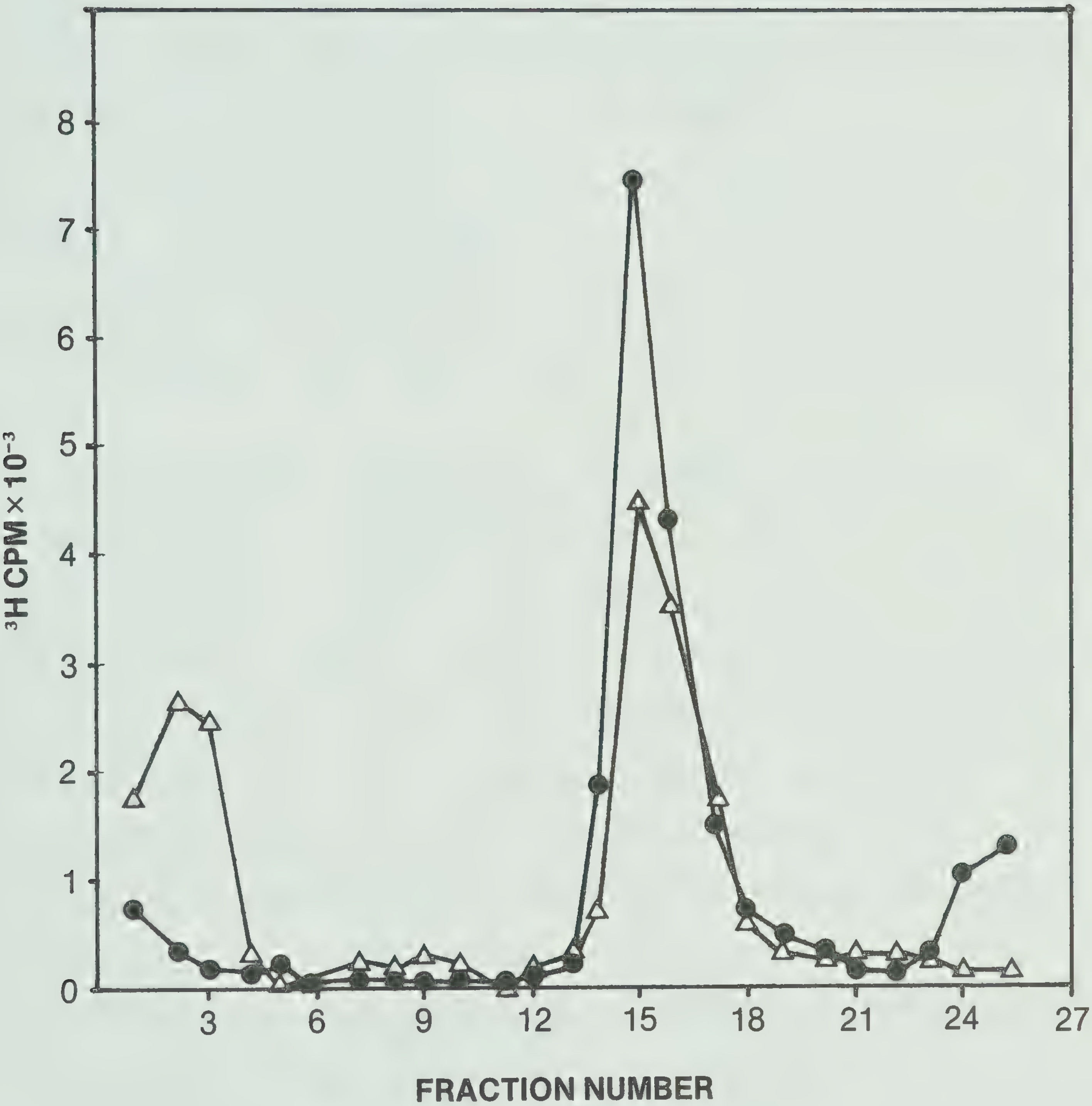
Figure 19

Virion Stability in Cesium Chloride

HeLa cells were infected with HAd4 ts1 at a MOI of 5 ffu/cell. The cells were incubated at 39°C and 32°C and labelled with (^3H)-thymidine at 20 and 40 h PI, respectively. The cells were then harvested 2 and 3 days after labeling at which time virus was freon-extracted. The resulting material was analyzed for the presence of complete virions by equilibrium centrifugation.

●-● (^3H)-thymidine counts/min incorporated at 32°C

△-△ (^3H)-thymidine counts/min incorporated at 39°C



8
7
6
5
4
3
2
1
0
^{3}H CPM × 10^{-3}
3
6
9
12
15
18
21
24
27
FRACTION NUMBER

study of the infected cells was carried out, with over 200 cell sections examined. Crystalline arrays of adenovirus-like particles were observed in the HAd4 ts1 infected cells, at both the permissive and non-permissive temperatures. Examination of the mutant infected cells, however, revealed 70-80% of the cells incubated at 39°C to exhibit an inefficiency in the production of large crystalline arrays at the restrictive temperature. In general, significantly fewer crystalline arrays were found in the nucleus of those cells grown at 39°C, compared to the amount present in both WT and HAd4 ts1 infected cells grown at 32°C.

The relative inefficiency of the HAd4 ts1 mutant to produce virion-like particles at 39°C, was further reflected by an apparent decrease in the overall activity present in the nucleus. On examination of WT infected cells, extensive viral activity was present as indicated by the presence of various types of viral inclusions, in addition to the crystalline arrays of virus. Inclusions bodies, Types I through V, as described by Shahrabadi, Marusyk, and Crawford (1977) were often seen in the nuclei of infected cells. These inclusions were also found in HAd4 ts1 infected cells grown at 32°C. The presence of such inclusions, however, was rarely observed in those cells infected with the HAd4 ts1 mutant and grown at the restrictive temperature. In most instances, a complete lack of these inclusions was apparent, although, occasionally, Type 1 inclusions were observed

(Figure 20).

No apparent difference in the size or structure of the virus-like particles produced by the HAd4 ts1 mutant at 39°C compared to that of the wild type was evident. In addition, all of the viral particles produced appeared to be mature as suggested by the presence of densely-stained cores (Figure 21).

Figure 20

Electron Micrographs of Thin Sections of HeLa Cells Infected with WT HAd4 and HAd4 ts1

HeLa cell monolayers were infected with HAd4 ts1 and WT HAd4 at a MOI of 5 ffu/cell. The infected cells were incubated at 37, 39 or 32°C for 16, 43 and 90 h, respectively. Following incubation the cells were harvested and prepared for thin sectionning as outlined in Methods and Materials.

Magnification: X 10,000

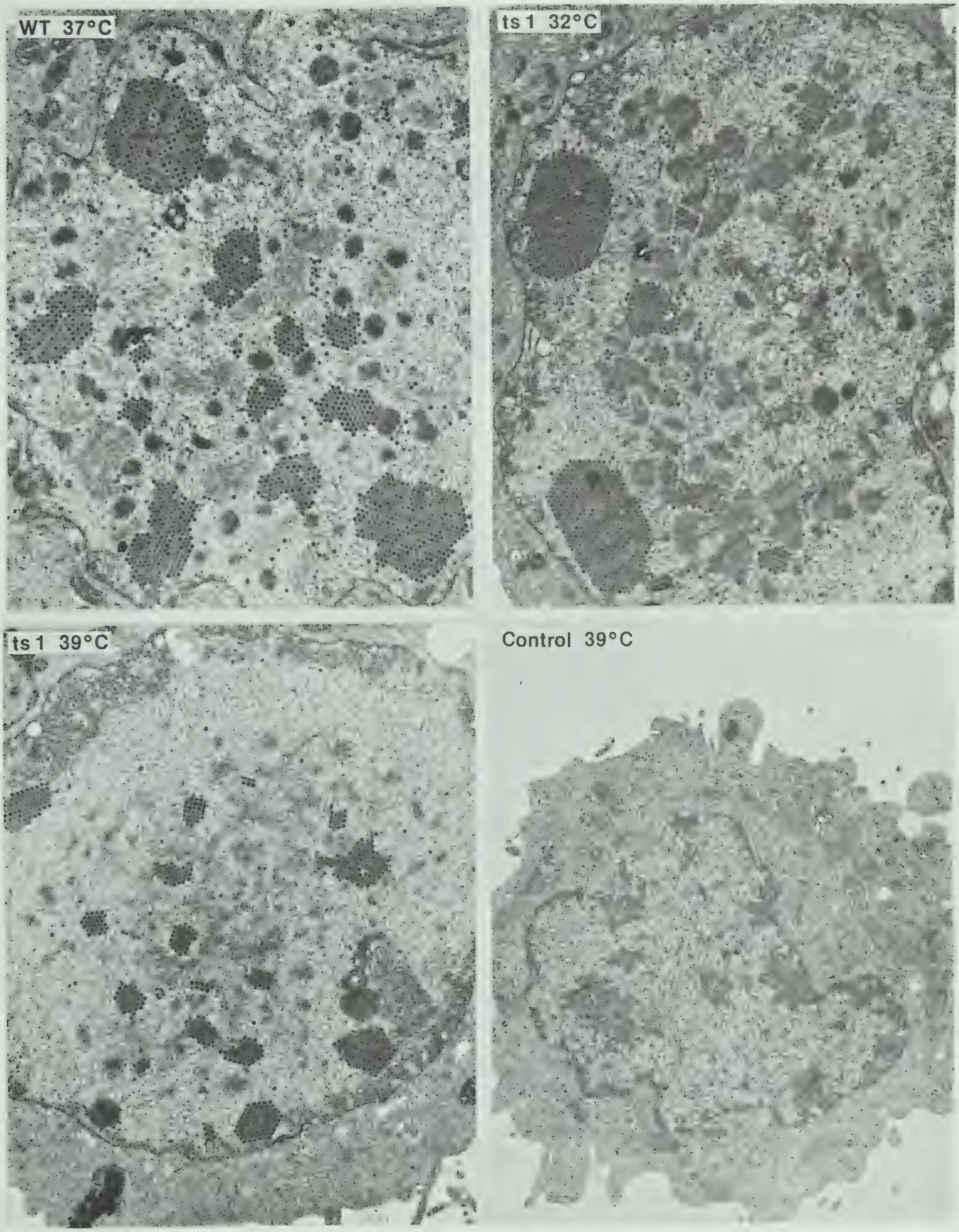
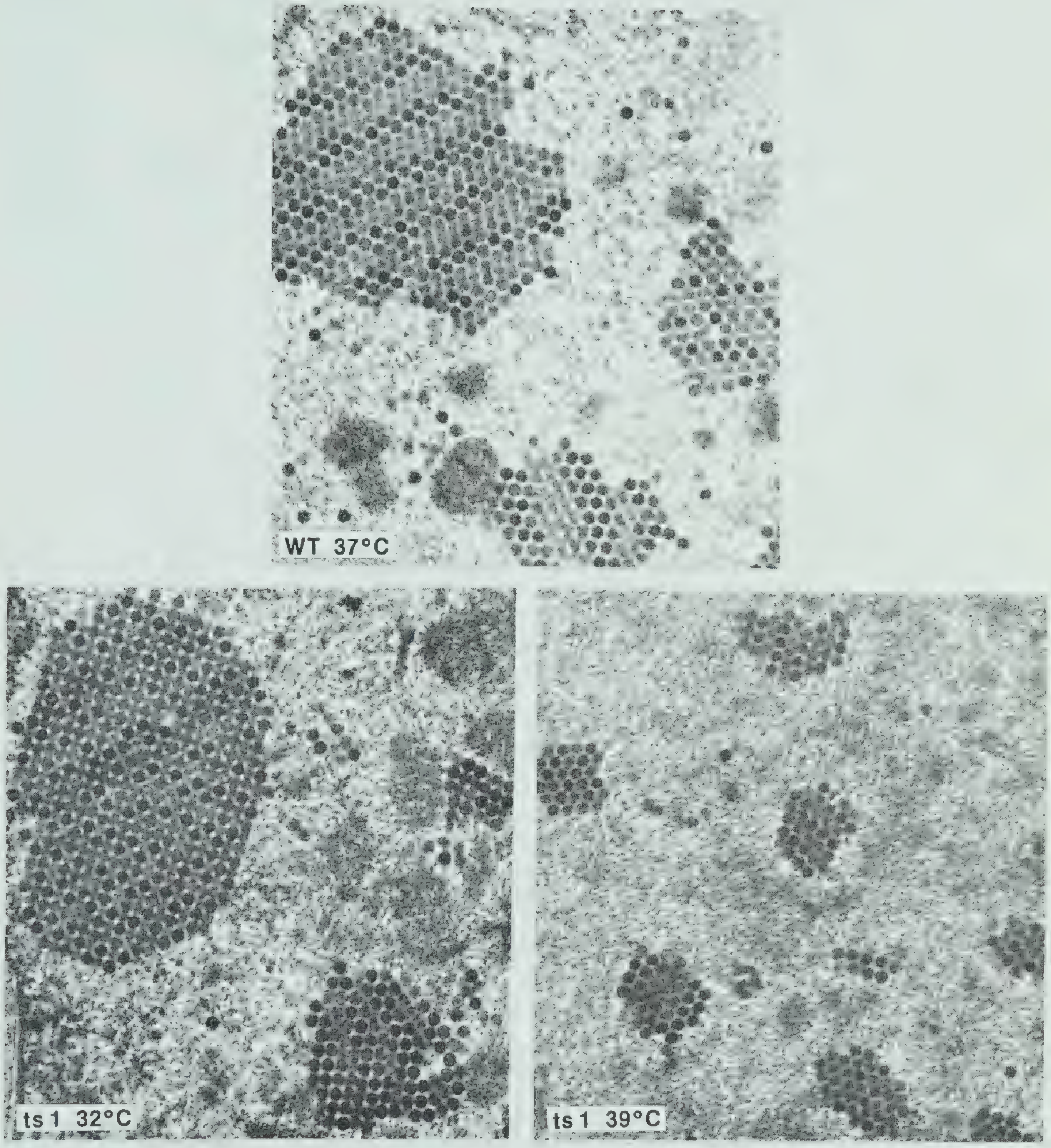

WT 37°C
ts 1 32°C
ts 1 39°C
Control 39°C

Figure 21

Comparison of Virion Morphogensis in Cells Infected with WT HAd4 and HAd4 ts1

Protocol followed was as described in Figure 20.

Magnification: X 25,000


WT 37°C
ts 1 32°C
ts 1 39°C

IV. DISCUSSION

In recent years, the isolation of virus conditional-lethal temperature-sensitive (ts) mutants has been reported by a number of authors (Ishibashi, 1970; Begin and Weber, 1975; Weber et al., 1975; Ginsberg and Young, 1977; Martin et al., 1978). The isolation of these ts mutants has permitted further investigation into the mechanisms regulating viral replication, as well as those involved in cellular function. In addition, the use of these ts mutants has allowed for some insight into the structure and functional role of certain of the viral gene products.

As mentioned previously, HAd4 occupies a unique position within the human adenovirus subgroup. The isolation of ts mutants of this subgroup is, therefore, of particular value in terms of elucidating the relationship of HAd4 to other members of the subgroup. The isolation of such HAd4 ts mutants, however, has not been previously reported. In this study, a ts mutant of HAd4 was isolated by nitrous acid mutagenization of wild type virus. The isolated mutant was designated HAd4 ts1 and was partially characterized by two-dimensional immunoelectrophoresis. The phenotypic character of this mutant was analyzed with particular reference to the ts mutants of other adenoviruses.

Since it was the intention of this study to analyze the HAd4 ts1 mutant by two-dimensional immunoelectrophoresis, it was first necessary to identify the antigenic pattern of WT HAd4 soluble component (SC) material. The two-dimensional

profile of this material, as shown in Figure 8, was found to exhibit four precipitin peaks which were identified as hexon, free penton base and the separate fiber and penton base structural components of the complete penton. A fifth precipitin line, that of the free fiber, was also tentatively identified. When compared against the two-dimensional immunoelectrophoresis profiles of both WT HAd2 and HAd5 SC material, no distinct difference in the antigenic pattern of the HAd4 SC material was observed, with the exception of the proposed free fiber peak (Boudin, Moncany, D'Halluin, and Boulanger, 1978; Tsang, 1978). In the former profiles, free fiber was found to exhibit a slower migration rate than that of the other soluble components. In contrast, HAd4 free fiber, as tentatively identified in this study, was found to migrate at an intermediate rate. The reason for the difference in the electrophoretic mobilities of the fibers is not known. However, it is possible that they may reflect differences in amino acid composition, hence net ionic charge.

The frequency of mutant isolation is dependent on three factors: the mutagen used, the method of selection and the criteria used in defining a mutant. In this study, both nitrous acid and hydroxylamine treatment were found to be inefficient in the production of ts mutants. A mutagenic frequency of less than 0.002% was observed following nitrous acid treatment and no mutants were isolated after treatment with hydroxylamine. This finding is in direct contrast to

that reported by other investigators. Mutant isolation at a frequency of 0.01 to 10% has been reported from stocks of WT HAd2 and HAd5 and occasionally a frequency of as high as 23% has been reported following nitrous acid treatment (Williams et al., 1971; Ensinger and Ginsberg, 1972; Begin and Weber, 1975). The discrepancy in isolating HAd4 ts mutants at a much lower frequency relative to that of other adenoviruses can possibly be attributed to the screening method employed in mutant isolation. Difficulties encountered in assaying HAd4 by the plaque assay method restricted the selection procedure to a random screening process which required location of infective foci within the cell monolayer by use of an inverted microscope. A high probability of overlooking infective foci would, therefore, be expected by this technique.

The inability of the HAd4 virus stock to produce distinct plaques is not understood. It is known that newly formed adenoviruses tend to adhere to the host cell and thereby restrict viral spread until late in the infection (Kjellen, 1961). This phenomen is not known to affect plaquing ability, however, other than lengthening the incubation period required for plaque formation. The plaquing ability of the adenoviruses has been found to be dependent on the adenovirus type as well as the assay conditions. It can only be assumed that for some reason, the conditions in the plaque assay system used were inhibitory to viral spread.

Preliminary identification of the HAd4 ts1 isolate as a mutant was determined by measuring the ability of the isolate to produce infectious virus at both the permissive (32°C) and non-permissive temperatures (39°C). A 32°C/39°C infectivity ratio of 10^3 was estimated for the HAd4 ts1 mutant. Viral clones exhibiting a three log difference in the ability to produce infectious virus at the permissive and non-permissive temperature are generally classified as being stable (Martin et al., 1978). By definition, these stable mutants have been found to exhibit low reversion frequencies, ranging between 10^{-3} to 10^{-6} (Ensinger and Ginsberg, 1972; Begin and Weber, 1975). This finding is in direct contrast with that of other DNA viruses, where a relatively high degree of leakiness or reversion has been observed in the ts mutants (Sambrook, Padgett, and Tomkins, 1966; Eckhart, 1969; DiMayorca and Callender, 1970). Although the reversion frequency of HAd4 ts1 was not determined, in view of the stability of other adenoviruses exhibiting a similar 32°C/39°C infectivity ratio, a relatively low reversion frequency would be expected with HAd4 ts1. In order to reduce the potential for reversion, however, all experiments were performed using HAd4 ts1 stocks with a passage history not greater than four.

The phenotypic characterization of the HAd4 ts1 mutant consisted of an analysis of viral replication with reference to the isolate's ability to produce soluble capsid components, synthesize DNA and exhibit normal viral

assembly. In addition, the heat stability of the mutant was examined as was the mutant's stability in CsCl.

The capacity of ts mutants to induce the synthesis of immunologically active capsid antigens has allowed for the subdivision of ts mutants on the basis of their respective phenotype. Three principle types of two-dimensional electrophoresis patterns have since been identified: a) those exhibiting a reduction or disappearance in one or several of the structural antigens; b) those exhibiting a general reduction in all of the viral antigens and c) those exhibiting an antigenic profile similar to that of the wild type (Russell et al., 1972a; Martin et al., 1978). The ability of the HAd4 ts1 mutant to synthesize immunologically-reactive soluble components was examined at both the permissive and non-permissive temperatures. Whereas normal antigenic capsid production was evident at the permissive temperature, a complete disappearance of three of the structural components, the complete penton, penton base and fiber, occurred at the non-permissive temperature. The synthesis of the hexon component appeared normal (Figure 13).

The two-dimensional immunoelectrophoresis profile of HAd4 ts1 was reminiscent of that of ts mutants of HAd2 and HAd12, which were also found to be defective in both penton base and fiber production/assembly (Shiroki, Irisawa, and Shimojo, 1972; Martin et al., 1978). Although the nature of the ts defect of these mutants was not reported, the

phenotypic characterization of the defect suggested that the defect was either in the genes responsible for capsid production or in the genes regulating their production.

Since the apparent production of the components of HAd4 ts1 was found to occur only at the permissive temperature, the effect of temperature-shift on the presence of the components was analyzed. Shift-up experiments to 39°C showed no detectable components to be present in the cell extract. This finding indicated that the nature of the defect was temperature-sensitive. In contrast to this finding, when the effect of temperature shift-down to 32°C was examined immunologically-reactive components were detected within 8 h following shift-down. A further increase in the amount of components was observed within 24 h (Figure 15). As previously stated, adenovirus replication is known to take approximately 16-24 h at 37°C, with synthesis of the structural components beginning by 14 h postinfection (Philipson et al., 1975). In addition, the growth curves of WT HAd4 indicate that a slower rate of replication occurs at 32°C compared to that at 37°C. In view of these facts, synthesis of the structural components by HAd4 ts1 at 32°C would be expected to occur significantly later than 14 h postinfection. The re-establishment of component synthesis (processing?) following 8-24 h of incubation at the permissive temperature indicates that the ts defect is not in the genes responsible for the synthesis of the components but more likely in a gene(s) responsible for the processing

of the components. This finding was further supported by the 1.8-fold increase in penton base over that of the mutant control at 32°C. The immunological detection of the components at the permissive temperature within 8-24 h following shift-down implies, therefore, that the nature of the defect is in some aspect of the processing of the components and not in synthesis.

The heat stability of mutant virions have been examined following exposure of virus to 50°C for variable time periods. In general, increased thermolability of ts mutants has been interpreted as suggesting the defectiveness of a structural component or a protein implicated in the morphogensis of the virion (Weber et al., 1975; Estes and Butel, 1977). A clustering of mutants into three groups, those indistinguishable from WT, those inactivated 10 to 50-fold faster than WT and those inactivated 1000-fold faster than WT has been reported (Weber,1975). On the basis of the thermolability profile of these mutants, it has been possible to place mutants exhibiting similar phenotypic defects into separate complementation groups (Weber et al., 1975). In this study , the heat lability of HAd4 ts1 was found to be increased 25-fold over that of the WT virus (Figure 16). This observation supported the previous finding of a regulatory defect in the processing of the structural components. Furthermore, the increased thermolability of the mutant and the apparent instability of the temperature-sensitive defect, irrespective of its

temperature of synthesis, suggests that the ts lesion may also result in an alteration in the maturation or assembly of the capsid components into the complete virion. Although the nature of the alteration was not determined the increased thermolability of the virions produced attests to its presence.

Viral DNA replication has been shown to be a necessary pre-requisite for the synthesis of viral capsid antigens (Flanagan and Ginsberg, 1962). This finding has since been supported by the observation that mutants defective in a late function have been shown to be capable of establishing viral DNA synthesis at the non-permissive temperature (Ensinger and Ginsberg, 1972; Wilkie et al., 1973). Differences in the rate of viral DNA replication, however, has been reported with different complementation groups of ts mutants (Suzuki et al., 1972). In view of the above findings, the ability of the HAd4 ts1 mutant to synthesize hexons (Figure 13) indicated that viral DNA replication occurred at the non-permissive temperature. In order to determine whether any difference in the rate of DNA replication was apparent at the permissive and non-permissive temperatures, analysis of DNA synthesis was examined (Figure 18). No significant difference in the rate of replication was apparent. The HAd4 ts1 mutant appeared to be equally capable of establishing viral DNA replication at both temperatures.

The assembly of virus by ts mutants defective in the

production of penton or penton base has been reported (Weber et al., 1975; Martin et al., 1978). Both HAd2 ts11 and ts7 mutants were found to be capable of assembling virus at the non-permissive temperature. Furthermore, the viral particles produced were found to be stable in CsCl gradients (Weber et al., 1975). Analysis of the HAd4 ts1 mutant gave similar findings (Figures 20 and 21). Initially, virus assembly was analyzed by electron microscopy of infected cells. Crystalline arrays of adenovirus-like particles were observed at both the permissive and nonpermissive temperatures. Further examination of the infected cells, however, revealed a reduced efficiency of the mutant to produce virus particles at the nonpermissive temperature. In addition, a significant reduction in the overall activity present in infected nuclei was observed; as indicated by the absence of viral inclusion bodies.

Further examination into the nature of the assembled viral particles at 39°C, showed them to be structurally stable in CsCl (Figure 19). The viral particles were also found to contain nucleic acid. As expected on the basis of the electron microscopy study, a 40% reduction in the assembly of virions was observed at the nonpermissive temperature. This finding is similar to that reported by Weber et al. (1975) whereby ts mutants defective in the synthesis of penton base were also found to exhibit reduced virion assembly at the nonpermissive temperature.

The ability of penton defective mutants to assemble and

be stable in CsCl gradients is not surprising in view of the experiments by Prage et al. (1970). In these studies, Prage demonstrated that virions lacking both pentons and peripentonal hexons were stable in CsCl gradients. Furthermore, these particles were capable of being visualized by means of electron microscopy.

The HAd4 ts1 mutant has been found to be defective in the synthesis of immunologically reactive complete penton, penton base and fiber, production of the hexon component being normal. On the basis of temperature-shift experiments, it has been possible to associate the temperature-sensitive defect with processing of the structural components. In addition, the increased thermolability of the mutant suggests that the defect also results in an alteration in the architecture of the complete virion, rendering it more heat labile. As with all late mutants, HAd4 ts1 was capable of establishing DNA synthesis at the non-permissive temperature. In addition, viral assembly was found to occur.

No significant difference in the characteristics of HAd4 ts1 was discerned with reference to properties of other ts mutants belonging to similar phenotypic classes. Penton and fiber defective mutants have been isolated from wild stocks of HAd2, HAd5 and HAd12 (Shiroki et al., 1972; Russell et al., 1972a; Weber et al., 1975; Martin et al., 1978). Although complementation studies were not done, the similar phenotypic characteristics of these mutants with that of HAd4 ts1 suggests that in at least some cases, HAd4

ts1 would be found defective in similar gene functions. Further information into the relationship of HAd4 ts1 to that of the other ts mutants requires that detailed intertypic complementation studies be done. Such studies would be essential in order to gain a further understanding of the unique characteristics of the HAd4 virion.

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